Novel approaches for the diagnosis of drug-resistance, treatment response, and infectiousness in patients with tuberculosis

(the eDIToR study – **D**iagnosis **I**nfectiousness and **T**reatment **R**esponse)

by

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Declaration

By submitting this dissertation electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the sole author thereof (save to the extent explicitly otherwise stated), that reproduction and publication thereof by Stellenbosch University will not infringe any third party rights and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

This dissertation includes one original paper published in a peer reviewed journal, one original paper under review in a peer reviewed journal, and three chapters of unpublished work in a format ready for submission (Chapters 4-6). The development and writing of the Chapters were the principal responsibility of myself. When applicable, a declaration is included indicating the nature and extent of the contributions of co-authors.

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"This isn't *magic*, is it?"

"I don't think so," said Johnny. "It's probably just very, very, very strange science."

"Oh, good," said Yo-Less. "Er… What's the difference?"

- Sir Terry Pratchett, Johnny and the Bomb

"But then science is nothing but a series of questions that lead to more questions, which is just as well or it wouldn't be much of a career path, would it?"

- Sir Terry Pratchett and Stephen Baxter, Long Earth

List of Abbreviations

ACI	Anderson Cascade Impactor
AMK	Amikacin
AMR	Antimicrobial resistance
AUC	Area under the curve
BCH	Brooklyn Chest Hospital
BDQ	Bedaquiline
CAP	Capreomycin
CASS	Cough aerosol sampling system
CE	Cartridge Extract
CF	Culture Filtrate
CFU	Colony forming units
COPD	Chronic obstructive pulmonary disease
C_q	Quantitation cycle
C_T	Cycle threshold
C_{Tmin}	Minimum cycle threshold
dCE	Cartridge extract from diamond-chamber
DCTB	Differentially culturable tubercle bacteria
DR-TB	Drug-resistant tuberculosis
DST	Drug susceptibility testing
DS-TB	Drug-susceptible tuberculosis
EDCTP	The European and Developing Countries Clinical Trials Partnership

EMA	Ethidium monoazide
EMB	Ethambutol
EPCFE	Exponential phase cell free extract
ETH	Ethionamide
EtOH	Ethanol
FDA	Food and Drug Administration
FQs	Fluoroquinolones
FT	FluoroType MTBDR
gMask	Gelatine Mask
INH	Isoniazid
IQR	Interquartile range
KAN	Kanamycin
LATE	Linear after the exponential
LCASS	Liquid cough aerosol sampling system
LPA	Line probe assay
LTBI	Latent TB infection
LZD	Linezolid
MDR-TB	Multi-drug resistant tuberculosis
MGIT	Mycobacteria growth indicator tube
MIRU	Mycobacterial interspersed repetitive units
MOX	Moxicillin
MPN	Most probable number assay

Mtb	*Mycobacterium tuberculosis*
MTBC	*Mycobacterium tuberculosis* complex
NAAT	Nucleic acid amplification test
NALC	N-acetyl-L-cysteine
NaOCl	Sodium hypochlorite (bleach)
NaOH	Sodium hydroxide
NGS	Next-generation sequencing
NHLS	National Health Laboratory Services
NPV	Negative predictive value
NRF	National Research Foundation
NTM	Non-tuberculous mycobacteria
OD	Optical density
OFX	Ofloxacin
PANTA	Polymyxin B, amphotericin B, nalidixic acid, trimethoprim and azlocillin
PB	Phosphate buffer
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pDST	Phenotypic drug susceptibility testing
PMA	Propidium monoazide
PPV	Positive predictive value
PZA	Pyrazinamide
qPCR	Quantitative polymerase chain reaction

RASC	Respiratory aerosol sampling chamber
RIF	Rifampicin
RNA	Ribonucleic acid
ROC	Receiver operating characteristic
RPF	Resuscitation promoting factors
rRNA	Ribosomal ribonucleic acid
RT	Room temperature
SAMRC	South African Medical Research Council
SEM	Standard error of the mean
SITVIT	*Mycobacterium tuberculosis* genotyping tool
SLIDs	Second-line injectables
SMOR	Single molecule overlapping reads
TB	Tuberculosis
TTN	Time to negativity
TTP	Time to positivity
TUB-band	Confirmation of presence of MTBC DNA
Ultra	Xpert Ultra MTB/RIF
UV	Ultraviolet
V4	Variable region 4 on 16S ribosomal RNA
VNTR	Variable number of tandem repeats
vPCR	Viable polymerase chain reaction
WHO	World Health Organization

WT	Wild type
XDR-TB	Extensively drug resistant tuberculosis
Xpert	Xpert MTB/RIF G4

Summary (Figure 1)

Drug-resistance tuberculosis (DR-TB) is a major challenge facing TB control. Limiting person-to-person transmission is key. This can be done by reducing time to drug susceptibility diagnosis and effective treatment initiation, which reduces infectiousness. Furthermore, DR-TB patients have suboptimal outcomes even on effective treatment and we have few methods for monitoring treatment response. If patients are not responding to treatment, better methods are required to measure infectiousness so that transmission may be limited.

First, to alleviate the under-diagnosis of drug-resistance stemming from additional sputa not submitted for drug susceptibility testing (DST) and infrastructural barriers, we showed that cartridge extract (CE) from used TB-positive Xpert MTB/RIF (Xpert) tests is directly usable for MTBDR*sl* (a second-line molecular DST). Furthermore, we showed that CE was useful for spoligotyping for molecular epidemiology.

Second, we showed that this CE approach is feasible on Xpert MTB/RIF Ultra (Ultra), which is Xpert's successor. We also evaluated the risk of *rpoB* amplicon escape during the extraction and the usefulness of material in other cartridge chambers for different molecular tests. In short, cross-contamination was possible but appears extremely unlikely. Only the diamond cartridge compartment contains useful material.

Third, MTBDR*sl* itself has limitations. For example, it only measures susceptibility to two drug classes. We assessed the feasibility of ultra-deep sequencing (single molecule overlapping reads, SMOR) on CE. SMOR had more actionable results (useful for clinical decision making) on Xpert CE than Ultra CE, and detected micro-heteroresistance missed by conventional DST.

Next, to evaluate the utility of new tools for treatment response, we leveraged a MDR-TB drug trial (NeXT, Clinicaltrials.gov #NCT02454205) to collect serial sputa. We assessed if sputa contained differentially culturable tubercle bacilli (DCTB) with a dormancy-associated

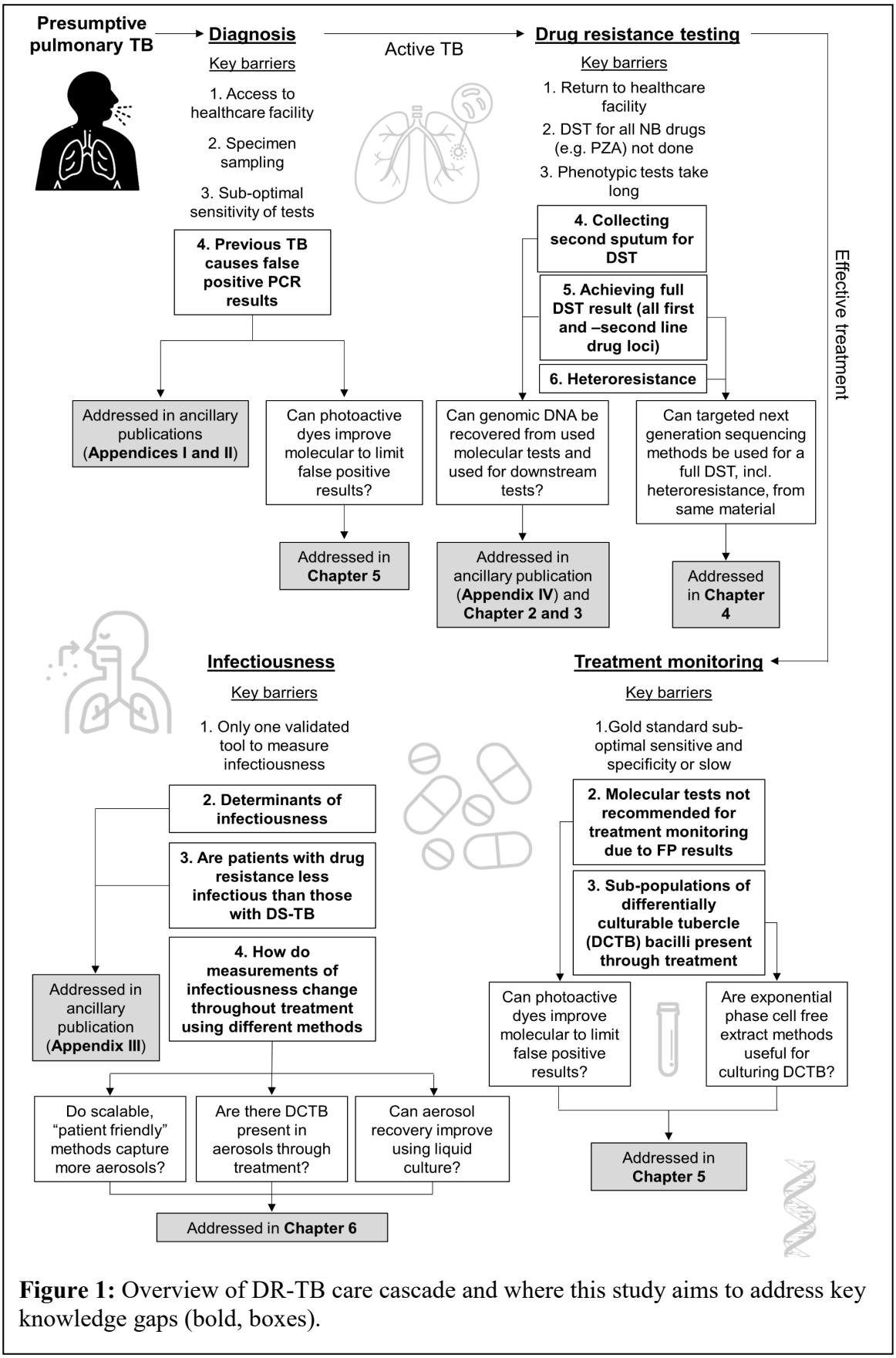

Figure 1: Overview of DR-TB care cascade and where this study aims to address key knowledge gaps (bold, boxes).

phenotype measured by the most probable number (MPN) assay. More bacilli were culturable with exponential cell free extract (EPCFE) and the MPN assay detected bacilli only culturable

with EPCFE in 22-30% of patients. As Xpert is confounded by DNA associated with dead bacilli, we also evaluated if Xpert for treatment monitoring is improved through use of a DNA-binding dye that blocks DNA amplification; however, the dye did not reduce Xpert-positivity.

Lastly, it is crucial to know when patients are non-infectious. New methods for detecting bacilli in cough aerosol were developed. First, to improve ease-of-use over the cough aerosol sampling system (CASS) reference standard, we designed a N95 mask containing a gelatine filter (gMask). Secondly we designed a liquid CASS (LCASS) to capture aerosol in liquid rather than solid media. gMasks showed patients remained infectious into treatment for longer than detected using CASS or LCASS, and that LCASS with EPCFE detected aerosol DCTB .

In summary, this work shows time-to-treatment initiation for XDR-TB could be reduced by doing second-line DST on CE from used cartridges. Targeted next generation sequencing on CE is feasible, but optimisation is required. Furthermore, we showed that EPCFE improved culturability (sputum and culture) by detecting patients with DCTB missed by conventional methods. New methods for measuring infectiousness indicated some patients remain infectious for longer than current paradigms suggest.

Opsomming

Dwelmbestande tuberkulose (DR-TB) is 'n groot uitdaging wat TB-beheer in die gesig staar. Ten einde die epidemie uit te roei, is dit nodig om die oordrag van person-tot-person te verminder. Dit kan gedoen word deur die tyd-tot-diagnose en tyd-tot-effektiewe behandeling te verminder, wat die duur van aansteeklikheid dus verminder. Boonop het DR-TB-pasiënte suboptimale uitkomste, selfs vir effektiewe behandeling, en daar is min goeie metodes om die die reaksie van die behandeling te monitor. As pasiënte nie op behandeling reageer nie, sal aansteeklikheid steeds verhoog word. Om oordrag te beperk, is beter metodes nodig om aansteeklikheid beter te bepaal.

Ons het eerstens getoon dat die “cartridge extract” CE) van gebruikte TB-positiewe Xpert MTB/RIF toetse toetse gebruik kan word om die onderdiagnose van DR-TB te verminder. Die onderdiagnose is a.g.v. die feit dat addisionele sputa nie vir medisyne-vatbaarheidstoetsing (DST) ingesamel word nie asook, die infrastruktuur beperkings wat met DNA toetsing gepaard gaan. CE is direk bruikbaar vir MTBDR*sl* ('n tweede-lyn molekulêre DST). Verder het ons aangetoon dat CE nuttig was vir akkurate spoligotipering vir molekulêre epidemiologie.

Tweedens het ons getoon dat hierdie benadering uitvoerbaar is op Xpert MTB/RIF Ultra (Ultra) CE, wat die opvolger van Xpert is. Ons het ook die risiko vir ontsnapping van *rpoB*-amplikon tydens die ekstraksieproses en die bruikbaarheid van CE van ander kamers in die Xpert in Ultra vir verskillende molekulêre toetse geëvalueer. Kortom, kruisbesmetting was moontlik, maar dit blyk uiters onwaarskynlik. Slegs die diamant-CE-gedeelte bevat nuttige materiaal.

Derdens het MTBDRsl beperkings. Gegewe hierdie beperkinge, het ons die uitvoerbaarheid van ultra-diepe-opvolging (“single molecule overlapping reads”, SMOR) op CE ondersoek. SMOR het meer uitvoerbare resultate (d.w.s nuttig vir kliniese besluitneming) op Xpert gehad as Ultra CE en het heteroweerstandigheid waargeneem wat deur konvensionele DST op isolate nie opgetel is nie.

Volgende, om vas te stel of nuwe metodes vir die monitering van behandeling nuttig kan wees, het ons gebruik gemaak van 'n MDR-TB-behandelings-ondersoek (NeXT, Clinicaltrials.gov # NCT02454205) om seriële sputa te versamel. Ons het beoordeel of sputa verskillende kultureerbare tuberkelbacilli (DCTB) bevat met 'n dormansie-geassosieerde fenotipe. Die aantal bacilli was gekwantifiseer me die “most probable number” (MPN) toets. Meer bacilli was kweekbaar met eksponensiële selvrye ekstrak (EPCFE) gedurende behandeling, en die MPN-toets het gevind dat 22-30% van bacilli slegs met EPCFE gekweek kon word. Aangesien Xpert verwar word deur DNA wat verband hou met dooie basille, het ons ook ondersoek of die gebruik van Xpert as ‘n behandelingsmoniteringsinstrument verbeter word deur die gebruik van 'n DNA-bindende kleurstof; die kleurstof het Xpert egter nie verbeter nie.

Laastens, is dit uiters belangrik om te weet wanneer pasiënte nie meer aansteeklik is nie. Nuwe metodes om TB-basille in hoes-aërosol op te spoor, is ontwikkel. Die eerste, wat die gebruiksgemak verbeter ten opsigte van die CASS-verwysingstandaard vir hoes-aërosolmonsters, was 'n N95-masker wat 'n gelatienfilter (gMask) bevat wat aërosol vasvang. Die tweede metode [vloeibare CASS (LCASS)] het aërosol in vloeistof eerder as vaste media vasgevang. gMasks het getoon dat pasiënte langer deur die behandeling aansteeklik bly as met CASS of LCASS, en dat LCASS met behulp van EPCFE DCTB in aerosol kan opspoor.

Samevattend wys hierdie werk dat die inisiëring van tyd tot behandeling vir XDR-TB kan verminder word deur tweedelyn DST op CE uit gebruikte cartridges te doen. SMOR op CE is uitvoerbaar, maar optimalisering is nodig. Verder het ons aangetoon dat EPCFE deur behandeling teenwoordig is end at dit die kweekbaarheid verbeter het deur pasiënte met DCTB op te spoor wat deur konvensionele metodes gemis is. Nuwe metodes om aansteeklikheid te meet, dui daarop dat sommige pasiënte langer aansteeklik is tydens behandeling as wat huidige paradigmas suggereer

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The thesis is in the hybrid format. It contains the following components in accordance with the Stellenbosch University Faculty of Medicine and Health Sciences guidelines for doctoral degrees. The key finding, the publication status, and the candidate's specific role is given for each Chapter below.

Candidate's role: Collecting Xpert cartridges, aliquoting and sorting of remnants. Collection of data.

Publication status: Published.

Mambuque E.T, Abascal E, **Venter R**, Bulo H, Bouza E, Theron G, García-Basteiro, A.L, García-de-Viedma G. Direct genotyping of Mycobacterium tuberculosis from Xpert MTB/RIF remnants. *Tuberculosis,* S1472-9792(18)30143-4, 2018. doi: 10.1016/j.tube.2018.05.008

Chapter 1

Introduction, Research Aims, and Originality

Introduction

The leading infectious cause of death worldwide is tuberculosis (TB), caused by the pathogen *Mycobacterium tuberculosis* (*Mtb*), which claimed 1.3 million lives in 2017[1]. TB is therefore a major focus of organisations such as the World Health Organization (WHO) and the Global Fund. Early diagnosis is one of the major challenges to reaching the End TB 2030 goals to eliminate TB. Although one focus of this thesis is the diagnosis of drug-resistant TB, there are, importantly, many challenges in the diagnosis of active TB too. These have been extensively reviewed to date[2].

1. Drug resistant tuberculosis

The emergence of drug-resistant (DR-) TB is major challenge that threatens to destabilise gains made in TB control. The burden of DR-TB ranges from 1% to 70% in some high burden countries[1] (Figure 1A). In South Africa, despite comprising a smaller percentage of the total TB burden, DR-TB consumes approximately 60% of the annual budget for TB management[3]. Similarly in the United States, MDR and XDR-TB were 8 to 25 times more costly than non-DR-TB patient[4].

Importantly, modelling studies[5] have shown that at least 80% of DR-TB is caused by person-to-person transmission (based on DR-TB incidence reports of high burden countries), rather than treatment-related acquisition of resistance (Figure 1B). This fact that transmission is the major driver of DR-TB is supported by house-hold contact and molecular fingerprinting studies showing that in high-burden countries such as South Africa and China, approximately 80% of DR-TB cases are likely due to primary transmission[6-9].

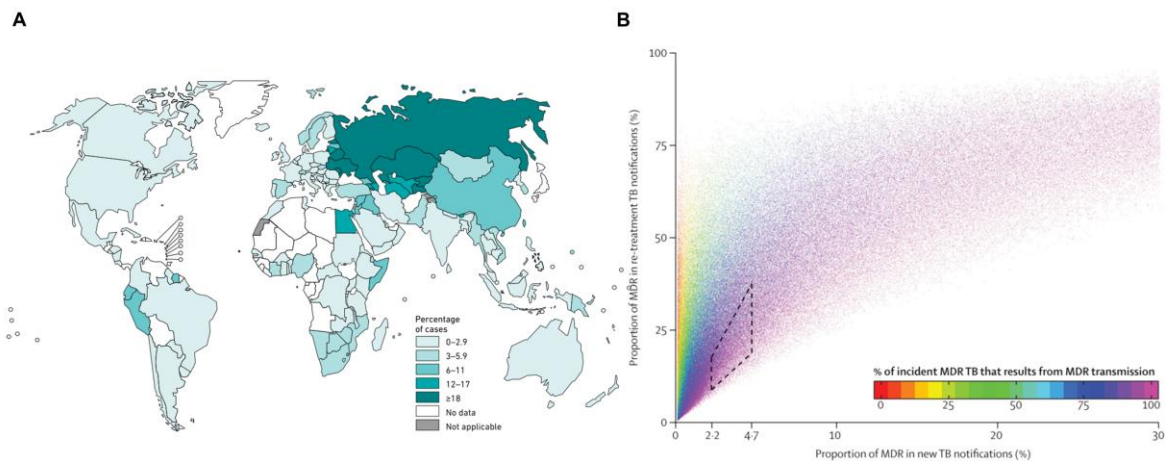

Figure 1: [A] Percentage of incident drug-resistant tuberculosis cases in 2017 amongst people who have TB for the first time[1]. **[B]** Estimated proportion of incident MDR-TB cases resulting from transmission (rather than acquisition during previous treatment in the same person). Current global notifications were consistent with a vast majority of MDR tuberculosis cases resulting from transmission[4].

Key to breaking the cycle of DR-TB transmission is starting patients on effective treatment earlier. This requires earlier diagnosis of drug-resistance. It is also important to identify patients with DR-TB who are not improving (treatment response) and have a high degree of infectiousness.

2. Drug susceptibility testing for drug-resistant tuberculosis

Only 40% of the estimated 558 000 incident cases of multi-drug resistant (MDR-)TB (resistance to the first-line drugs rifampicin and isoniazid) are reported. Worryingly, studies have shown that due to gaps in the TB care cascade in high burden countries like South Africa and India, only 20-40% of reported DR-TB cases are successfully treated (Figure 2A and B)[1,10,11]. Of the 558 000 MDR-TB cases, 8.5% are estimated to be extensively drug-resistant (MDR plus resistance to fluoroquinolones and a second-line injectable agent; XDR)[1]. XDR-TB is associated with especially poor outcomes. For example, studies have shown in KwaZulu Natal, South Africa, 11-90% of patients with XDR-TB died before treatment initiation and 36% of patients who were treated died at follow-up[12,13]. Patients with DR-TB are also often discharged back into communities despite being possibly infectious[14].

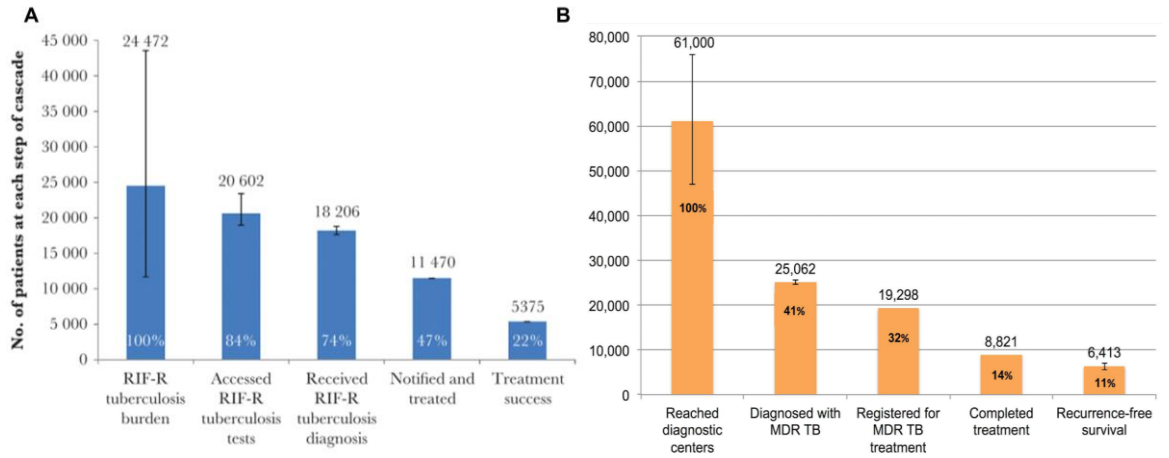

Figure 2: [A] Care cascade for patients with at least rifampicin-resistant (RIF-R) tuberculosis. In South Africa[3] and **[C]** India[4]. Many patients with at least rifampicin-resistant tuberculosis are not diagnosed, started on effective treatment or have treatment success.

There are several reasons for this gap in the care cascade, one of which is the need for collection of a second sputum. According to WHO-recommendations, national algorithms dictate that once TB diagnoses are confirmed and rifampicin-resistance suspected this second sputum is collected and used for additional drug-susceptibility testing[15,16]. However, patients often do not return for a follow-up visit, due to various reasons (e.g. transport, time off work, travel costs, too ill.), and treatment initiation has been shown to be delayed as long as 55 days[17], in which a patient may still be infectious. Some patients also struggle to produce a second sputum, especially in countries with a high HIV co-infection rate, which further limits additional specimen collection[18-21].

2.1. Current methods for drug-susceptibility testing

Xpert MTB/RIF (Xpert) is a nucleic acid amplification test that detects *Mycobacterium tuberculosis* complex DNA (MTBC) and resistance to rifampicin, a first line anti-TB drug, directly from sputa and is endorsed by the Food and Drug Administration (FDA) and WHO[22-24]. The latest data from 2016 shows that over 25 million Xpert cartridges have been bought and consumed worldwide[25]. It is an easy 3 step process that requires the addition of sample reagent to a sputum, loading the sample reagent-sputum mix into an Xpert cartridge and running this on the GeneXpert platform (Figure 3)[24].

Figure 3: Workflow of Xpert cartridge preparation. Takes approximately 2 min of hands on time and 2 hours run time.

Several national algorithms, with recommendation from the WHO, state that if a patient is tested with Xpert and rifampicin resistance is detected, a second sputum is to be collected for drug-resistance testing (DST) for resistance to isoniazid, fluoroquinolones and second-line injectables[16,24,26,27]. These tests are done either phenotypically, which involve waiting on cultures for up to 42 days or done using molecular methods such as the WHO-endorsed line probe assays (LPAs) namely, MTBDR*plus* (rifampicin and isoniazid) and MTBDR*sl* (fluoroquinolones and second-line injectables). These are done on sputa but requires certain biosafety and laboratory infrastructure for DNA extractions form the sputa. Suboptimal sensitivity on sputa have also been shown and thus LPAs sometimes needs to be done on the culture which adds further delays[28-31]. Despite national algorithms mandating the use of LPAs

for DST, poor adherence has been reported[32-34], for example, in South Africa, 34% of Xpert RIF-resistant patients failed to receive MTBDR*plus* and, of those confirmed to have MDR-TB, 28% did not receive second-line DST[21]. Novel approaches to reduce this gap in the TB care cascade is therefore a major research priority[35,36]

2.2. New tests for drug-susceptibility testing

Xpert has recently been superseded by an improved molecular test, Xpert MTB/RIF Ultra (Ultra) which also detects for MTBC and rifampicin resistance but has increased sensitivity for paucibacillary samples (though specificity is overall reduced) due to the addition of two different multicopy amplification targets (IS6110 and IS1081) and the use of melt-curves to detect resistance and improved *rpoB* probes[37-39]. Furthermore, a new first-line molecular DST test, the FluoroType MTBDR (FT), which is a non-symmetric [linear after the exponential (LATE)] PCR together with lights-on/lights-off probes has been designed and tested in clinical settings[40]. It has been designed as an automated qualitative test for detection of MTBC-DNA as well as resistance to rifampicin and isoniazid. Importantly it also gives the resistance mediated mutation associated with the resistance calls. It has been shown to have high sensitivity and specificity on clinical samples[41,42]. However, unlike the LPAs, the FT has not been endorsed by the WHO.

2.3. Heteroresistance and drug-susceptibility testing

The phenomenon of heteroresistance is becoming more prevalent. Heteroresistance can either be polyclonal – mixed-infection of two separate strains or the emergence of a sub-population of very rare, spontaneous DR-TB mutants in the presence of an antibiotic; or monoclonal – heterogeneity that occurs when one pure clone differentiates into drug-susceptible and drug-resistant populations at a high frequency. The former is more common (Figure 4)[43-47]. Polyclonal heteroresistance is more difficult to detect on pure clones from these samples as

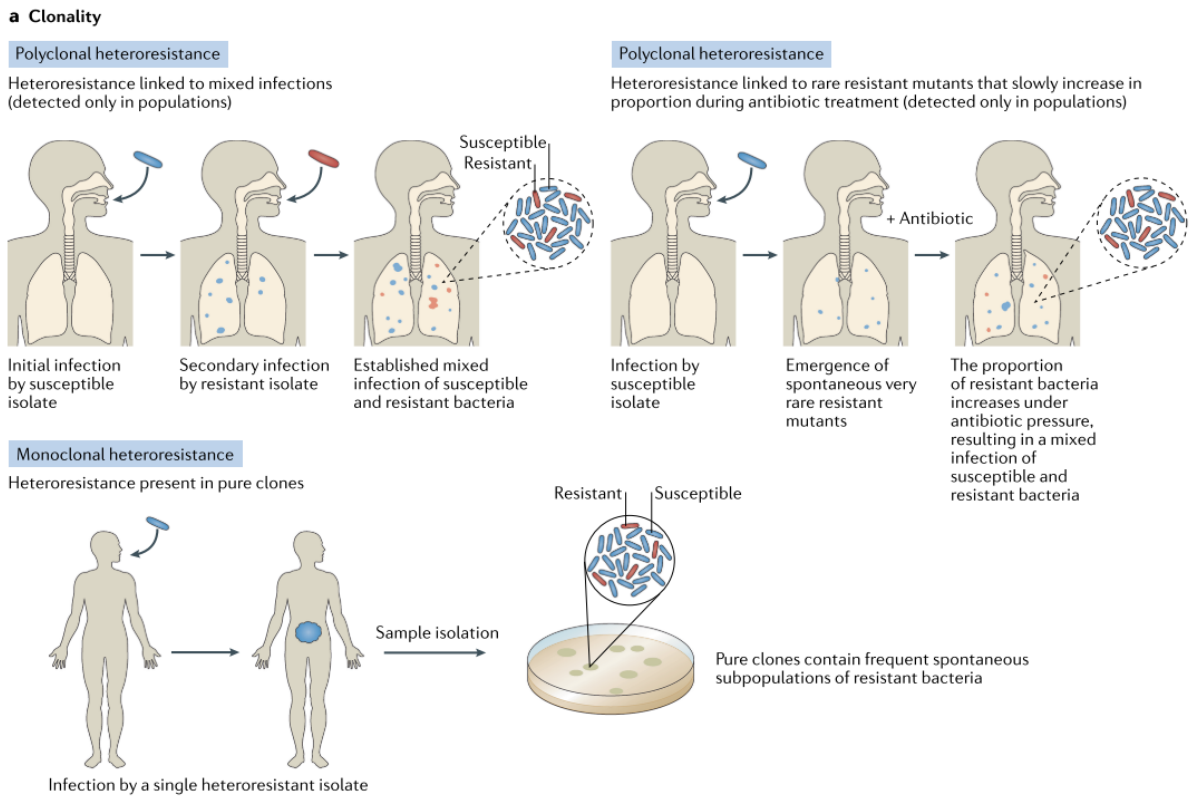

Figure 4: Clonality of heteroresistance. Heteroresistance can either be polyclonal (top panels) which exists as result of mixed infections or from rare resistant mutants that slowly increase in proportion during antibiotic treatment in a population of susceptible bacteria. It can also be monoclonal, which is generated from a single clone that differentiates into two populations (susceptible and resistant) at high frequency in the absence of antibiotic pressure[40].

they would either be fully resistant or fully susceptible during culturing[47]. Heteroresistance to both first –and second line drugs for TB has been reported, and these are often missed by conventional DST methods. Genetic diversity of *Mtb* is often lost during phenotypic DST as sub-culturing causes selection bias, especially in polyclonal heteroresistant cases[47-49]. Furthermore, molecular assays such as Xpert and LPAs often miss heteroresistant population as they are present at lower frequencies and are masked by the larger population of drug-susceptible bacteria or only detect certain resistance conferring mutations[50]. The misdiagnosis of these can lead to poor patient outcomes and better tools are necessary to ensure patients are on effective treatment[51].

2.4. Next generation targeted deep sequencing

A promising new method for a near-complete drug-resistance profile is the use of next generation sequencing methods such as targeted deep sequencing[45,52]. This will allow a complete antibiogram and detect proportions of resistant alleles down to f <1%. This is especially important for the detection of heteroresistance[45,49]. One such technology is the single-molecule overlapping reads (SMOR) method, which was developed and patented by The Translational Genomics Research Institute, Northern Arizona University which has been shown to obtain fast and accurate drug resistance profiles for both first and second-line anti-TB drugs[53-55].

2.5. Knowledge gaps for drug resistance testing

We aimed to address the above gaps by determining whether MTBC-DNA can be recovered from used Xpert and Ultra cartridges - that would otherwise have been discarded - and whether this could be used, without further DNA extraction methods, to generate a complete first and – second-line DST result from one sample. We aim to do this firstly using routine molecular DST methods as well as making use of SMOR.

3. Treatment response monitoring

Not only do we need to find ways to improve the diagnosis of DR-TB, but with large numbers of patients who default or have treatment failure, proper tools for monitoring treatment response in DR-TB patients are paramount. This would aid in regimen change and in-patient hospital management, for example, if we could detect at an early stage if a patient is likely to fail treatment, certain interventions could be taken to reduce the chance of this happening, thereby potentially improving outcomes. Current techniques such as culture and smear, which are slow, have relatively poor sensitivity and specificity, 24-57% and 81-85%, for predicting relapse and treatment failure respectively, even when performed several times

longitudinally[56,57]. Molecular diagnostics such as Xpert also has limitations such as the inability of the test to distinguish between DNA from live and dead bacteria.

3.1. Culture methods for treatment monitoring

A widely used standard for treatment monitoring is culture and smear, where if a patients is culture and smear negative it suggests that treatment is effective, however smear has low sensitivity and culture can take up to 42 days for a result and does not quantify the number of bacteria present[56]. There has also been several studies that indicate that there is a sub-population of differentially culturable tubercle bacteria (DCTB) in a dormancy-related state and that are drug-tolerant, which in turn allows them to proliferate through treatment. This sub-population has not yet been studied in terms of their association with treatment outcomes [58,59]. As these DCTB cannot be quantified using standard cultures (they often fail to grow), a modified culture method has been developed where resuscitation promoting factors (RPFs) or exponential phase cell free extract (EPCFE) from growing TB strains (which contain both RPFs and other rescue pathway proteins) are added during culture to promote growth[58-61]. The Most Probable Number (MPN) assay is a method that can be used to quantify the number of bacteria present in a starter culture through a serial dilution and has often been in microbiology, it can also be used to monitor the effect of one media against another[61,62].

3.2. Molecular tests for treatment monitoring

Xpert is a current WHO-endorsed test for TB diagnosis. Part of how the Xpert works that bacilli (not necessarily alive or intact) are trapped on a filter and washed with a wash fluid prior to DNA extraction via sonication (Figure 5A)[24,63]. This step is meant to remove any extracellular DNA, however, we have shown in our previous work it does not remove DNA-associated debris or DNA from non-viable cells (Figure 5B) and can lead to false-positive results, due to the bactericidal effects of the TB drugs or in in patients with previous TB (*although the candidate contributed significantly as a co-author to two publications on the topic of false-*

positive PCR results in previously-treated patients it is not the primary focus of this thesis and hence not discussed extensively; these publications are in Appendices I and II).[64-67]

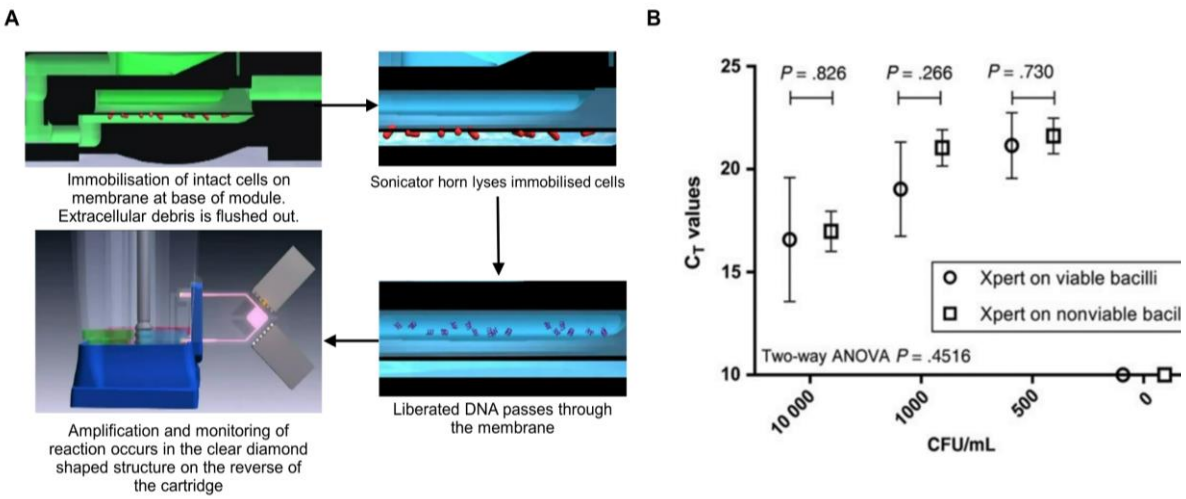

Figure 5: [A] Manufacturer-supplied graphics showing that bacteria (viable or non-viable) are trapped on a filter and washed before DNA extraction (via sonic horn) to remove any extra-cellular DNA[56] as part of the Xpert test. **[B]** However, this does discriminate between alive or dead bacilli: a comparison of Xpert cycle threshold values (C_T; mean ± SEM) from a dilution series of bacilli showed similar C_T values when Xpert was performed on intact bacilli or non-viable, heat- and mechanically-lysed bacilli[59].

3.3.1 Photoactive dyes used for viable PCR (vPCR)

Photo-reactive dyes, such as propidium monoazide (PMA) and ethidium monoazide (EMA) have been used to intercalate free DNA or DNA from non-intact cells by forming covalent bonds upon light-exposure; thereby preventing this DNA from being amplified. This technique is called viable polymerase chain reaction (vPCR)[68-71]. One of the limitations however are that some decontamination procedures can induce cell death while keeping cell membranes intact, in which case PMA and EMA have reduced sensitivity[72,73]. However, a novel dye, PEMAX, designed by GenIUL has been created to overcome this limitation[74]. It has been shown by Friedrich *et al* that dead DNA that accumulate through treatment because of the TB drugs confounds Xpert and thus Xpert remains positive further into treatment which makes it a poor diagnostic for treatment monitoring[64] (Figure 6). The use of vPCR together with Xpert may improve the specificity of Xpert for the detection of viable bacilli and thus reduce increase the

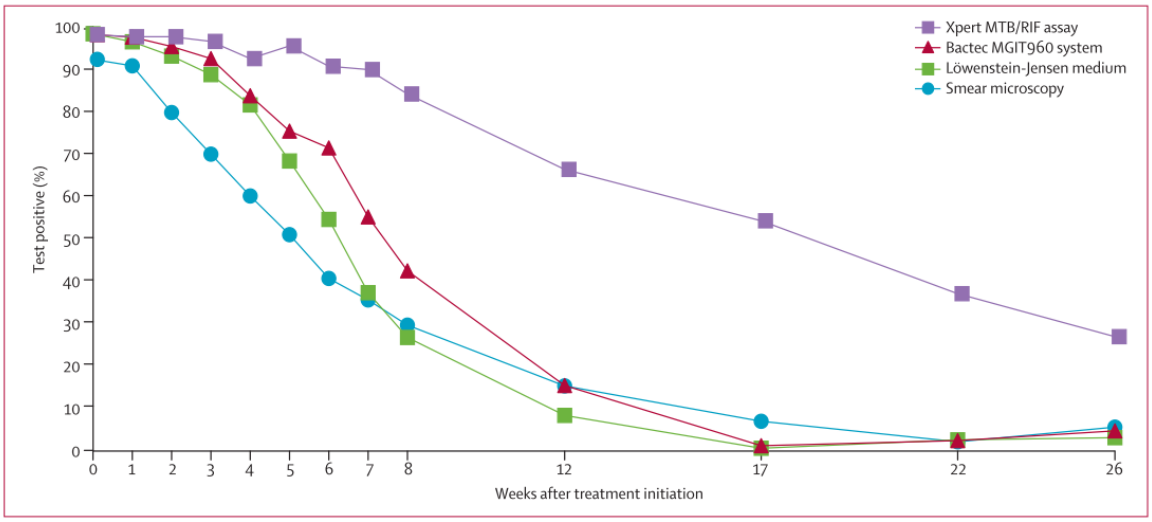

Figure 6: Data from patients for culture (Lowstein-Jensen medium and Bactec MGIT 960), smear microscopy and Xpert from baseline and at follow-up visits through treatmet. The proportion of positive tests for Xpert delines at a slower rate throughout treatment compared to culture and Xpert, likely due to detection of dead DNA[64].

diagnostic accuracy for monitoring treatment. A study was done in South Africa where they looked at the effect of anti-TB drugs (isoniazid, rifampin, pyrazinamide, ethambutol, streptomycin, moxifloxacin) on the detection of TB with Xpert both *in vitro* and on clinical specimens and how PMA could improve TB detection by Xpert. They did not however, look at the effect of new anti-TB drugs such as bedaquiline and linezolid. The study showed that treatment of sputum with PMA increases the specificity of Xpert and reduces false-positivity but only by a small measure based on which antibiotic is used during treatment[75].

3.3. Knowledge gaps for treatment monitoring

We therefore aimed to improve routinely used diagnostics for treatment monitoring, especially in patients with DR-TB. We did this by using the MPN assay to detect and quantify DCTB from patients enrolled in a longitudinal drug-trial comparing standard MDR-TB treatment to the new regimen containing both bedaquiline and linezolid. Furthermore, we aimed to improve on Xpert for treatment monitoring by pre-treating sputa from the same patients with the new PEMAX double-dye, to reduce the rates of false-positives through treatment.

4. The infectiousness of TB patients of treatment

It is important to improve on diagnostics to ensure patients are placed, and stay, on effective treatment as this reduces the risk that a person remains infectious. This is especially important for DR-TB given person-to-person transmission is the major driver of transmission (Figure 1A). The current paradigm is that patients who are smear-positive (detectible acid fast bacilli in their sputa) are more infectious and that those on effective treatment who convert to smear negative status and are no longer infectious, however, there is little data on this for patients with DR-TB[76-78]. Furthermore, we have recently shown, in a large study (n=500) looking at the determinants of infectiousness, that despite the current paradigm that patients with DR-TB are less infectious than those with DS-TB, almost half of patients with DR-TB, after taking into account treatment duration, were infectious using the CASS method, and that some were repeat positive, despite likely effective treatment. However, even though patients who were positive were sampled again, patients were not sampled longitudinally throughout treatment (*the candidate was a co-author of this output, which is included in Appendix III*).

It is therefore important to have tools to measure the infectiousness of patients, especially longitudinally, to better understand transmission, reduce the DR-TB burden, and inform on regimen changes and hospital discharge management strategies.

4.1 Tools for measuring infectiousness

4.1.1 Sampling on solid media (agar)

The Cough Aerosol Sampling System (CASS) is a technology that was developed to capture aerosol particles from patients with TB and quantifies both the size and distribution of individual particles by incorporating a six-stage Anderson Cascade Impactor (ACI)[79]. This system works by having a patient cough into a machine containing an ACI which houses 7H11 agar plates on six stages, each stage has varying size distribution and makes use of the inertia of the particles to separate them according to size. After a sampling time of 5 min, the machine

is taken to a Bio Safety Level 3 laboratory where the agar plates are removed, incubated for up to 6 weeks and the colonies counted.

The CASS has been used as a proxy measure for infectiousness in several house-hold contact studies and has been shown to predict transmission better than standard smear microscopy methods[80-82]. Although it is the only validated tool for measuring infectiousness it does have some limitations, namely that it is a large instrument that requires intensive infrastructure which limits its use in field settings or in larger studies and it is dependent on a short spell of forced coughing. Finally, it depends on solid culture where previous studies have shown that liquid culture is more sensitive for TB culturing[83-85].

4.1.2 Sampling in liquid media

Aerosol sampling using liquid has been validated before in other bacteria such as *Legionella spp*.[86] and *Campylobacter spp.*[87] as well as for the influenza virus[88-90] using SKC BioSamplers, but has not been reported for TB bacteria. It has the advantage of both using culturing in liquid, which as mentioned before has proved to be more sensitive for TB bacteria, but can also be used for molecular assays such PCR which allows for rapid identifications. Furthermore, it can be used in combination with various culturing media, such as the MPN containing broth supplemented with EPCFE which promotes growth of DCTB[58,91].

4.1.3 Face mask sampling

Another novel method to measure infectiousness includes using a mask altered to contain a gelatine filter which traps aerosolised *Mtb* particles. The filter can then be solubilised and the particles recovered[92]. This has been used to study patients with chronic obstructive pulmonary disease (COPD)[93] as well as in patients with TB both in the UK as well as in Pretoria, South Africa, however not on patients with DR-TB nor longitudinally[92,94] A major advantage of this

method is that it is portable, relatively inexpensive compared to solid agar or liquid broth methods and it does not depend on a short periods of forced coughing.

4.2 Knowledge gaps for measuring infectiousness

We aimed to address some of the limitations of the standard CASS by developing a method for capturing aerosols in liquid media from DR-TB patients. We also included culturing with EPCFE to determine if there were DCTB in aerosols during longitudinal sampling. Furthermore, we designed a mask containing a gelatine filter which can be solubilised to detect of there were culturable bacteria in the captured cough aerosol. These would both measured with CASS as reference standard, including longitudinally.

5. Study rationale and concluding remarks

In summary, while efforts are being made to reduce incidence rates and the number of deaths caused by TB, the emergence of drug-resistant TB threatens that goal and is a public health crisis. In order to better understand and control the drug-resistance epidemic many steps need to be taken. This includes strengthening health system, finding new TB drugs, and reducing transmission of drug-resistant TB, among others. In order to address the latter we examine ways to reduce the gap in the TB care cascade by finding ways to place people on effective treatment sooner, thereby reducing the time they remain infectious. We have also looked at ways to improve current diagnostic methods for treatment monitoring to ensure patients remain on effective treatment, and finally will develop and test new tools to measure the infectiousness of patients throughout treatment to determine if placing patients on effective treatment does reduce their infectiousness.

Summary of knowledge gaps and aims (Figure 7)

While there are many factors contributing to the DR-TB problem, there are several key knowledge gaps that are a focus of this thesis that, if answered, may help to reduce the burden of DR-TB (Figure 2). These gaps include: 1) improving diagnosis of DR-TB to ensure patients are started on effective treatment sooner 2) improving diagnostics for treatment monitoring to ensure patients remain on effective treatment and 3) discovering novel ways of measuring the infectiousness of patients to determine when a person is truly non-infectious. We therefore aimed to address these three main questions by finding ways to improve diagnostics for treatment initiation and treatment monitoring as well as discovering new tools to measure infectious throughout treatment

Aim 1: To investigate whether used Xpert MTB/RIF and Xpert MTB/RIF Ultra cartridges contain salvageable genomic DNA useable for further testing including genotypic DST, strain-typing and next generation sequencing.

Sub-aim 1a:

To use the cartridge extract (CE) from the diamond-shaped protrusion on the back of used Xpert MTB/RIF (Xpert) and Xpert MTB/RIF Ultra (Ultra) cartridges as well as other chambers of the cartridge to determine if *Mtb* genomic DNA remains and use this as a template for MTBDR*plus*, MTBDR*sl* and FluoroType MTBDR to determine both diagnosis and drug susceptibility testing (DST) from one specimen within 24 hrs.

Sub-aim 1b:

To determine if CE from used cartridges can be used as a template for molecular strain typing methods such as spoligotyping.

Sub-aim 1c:

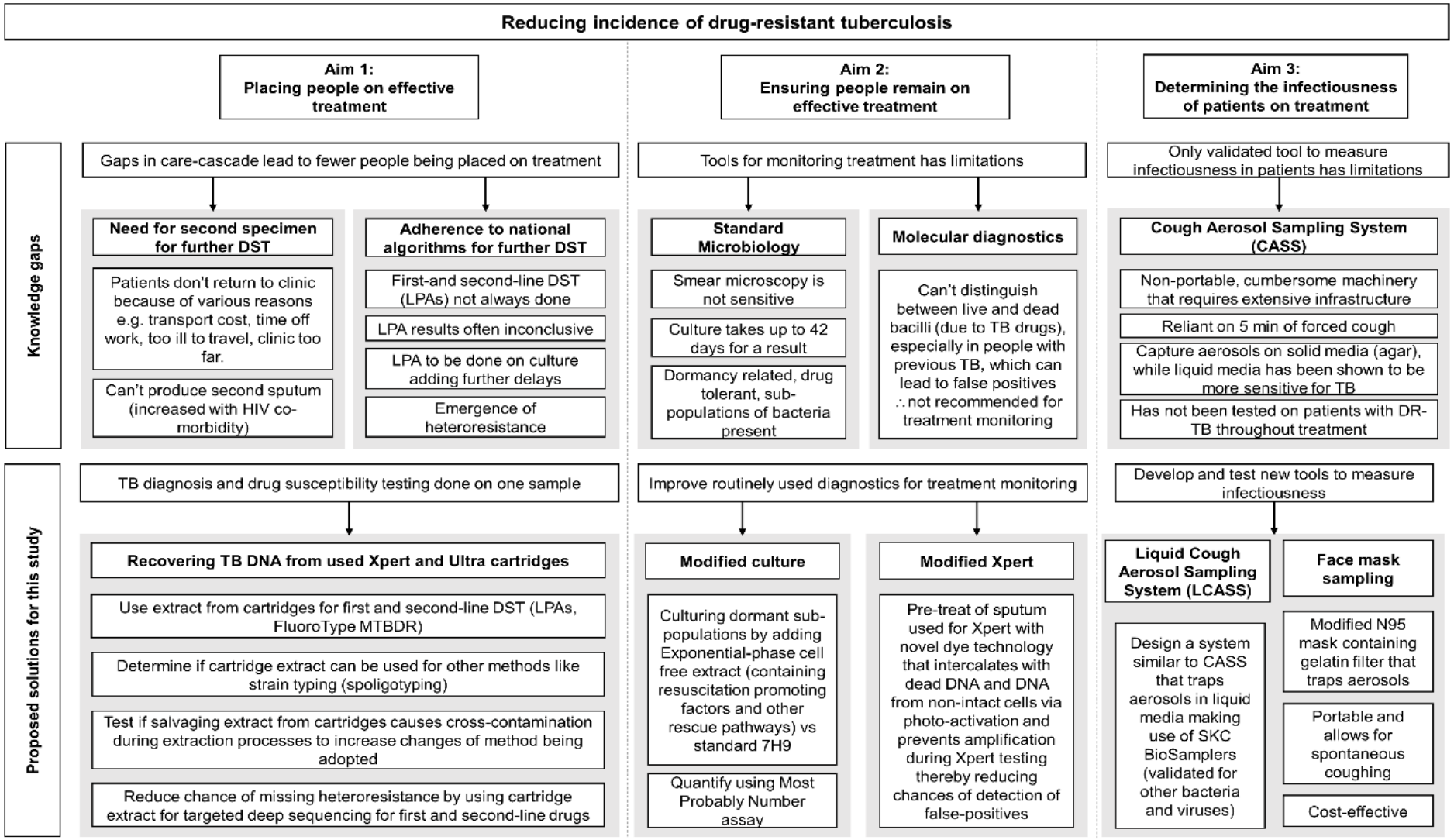

Figure 7: Overview of key knowledge gaps, the study aims to address them, and the key methods involved. tuberculosis.

To determine if CE from Xpert and Ultra is feasible as template DNA for next generation sequencing methods such as single molecule overlapping reads (SMOR) to get a full drug-resistance panel result from both first and second-line drugs and to see if there are any heteroresistance markers missed by conventional DST methods.

Aim 2: To determine if modified routinely available diagnostics, Xpert-vPCR and culture with added exponential phase cell free extract, are more sensitive for treatment monitoring compared to standard Xpert and culture as reference standards.

Sub-aim 2a:

To use a photo-activated dye technology combined with Xpert to prevent the amplification of DNA from non-viable and non-intact cells by comparing cycle threshold values generated from on clinical specimens to improve the sensitivity of Xpert.

Sub-aim 2b:

To use the MPN assay to monitor whether exponential phase cell free extract added to sputum from patients on treatment results in improved culturablity of a sub-population of bacilli in a dormancy related state.

Aim 3: To compare of new methods of cough aerosol sampling (liquid sampling, gelatine mask) for quantifying the aerolised bacterial load of patients with DR-TB on both standard-of-care regimen and the new MDR-TB regimen (incl. bedaquiline and linezolid) through treatment, compared to a reference standard using the solid-agar ACI-based CASS.

Sub-aim 3a:

To validate the liquid cough aerosol sampling system (LCASS) by capturing respirable cough aerosol droplets (<5µm) directly into liquid medium.

Sub-aim 3b:

To determine using the MPN assay containing exponential phase cell-free extract can be used to culture differentially culturable cells from aerosols captured in liquid media using the LCASS.

Sub-aim 3c:

To sample the cough aerosol of DR-TB patients using a mask containing a gelatine filter (which traps aerosolised bacilli) and to compare rates of positivity to both LCASS and CASS done on the same patients.

Originality of study

No studies have previously looked at salvaging genomic *Mtb* DNA from the PCR-reaction mix in the diamond chamber at the back of the cartridge. There has been two studies which used the remnants (sample reagent-and-sputum mix added to the cartridges) for further testing, (*for one of which the candidate is a co-author which can be found in Appendix IV*)[95,96]. While this is a useful approach it requires additional DNA extractions steps and has to be done in timeous manner as the sample reagent prolonged exposure to sample reagent can degrade DNA and potentially introduce mutations[24,97].

Previous studies have examined the utility of dyes such as EMA and PMA but none have done so systematically with longitudinal time points, have employed a rigorous microbiological reference standard. Two studies have tested the validity of PMA on *Mycobacterium tuberculosis*, one of which tested the effect of treating sputa from patients up until 12 weeks after treatment initiation with PMA prior to Xpert testing[68,75] (29, 33). However, neither of the studies made use of the novel double dye technology (PEMAX – comprising of both EMA and PMA, GenIUL) nor on longitudinal sputum samples from patients that are currently on the new MDR-TB regimen of bedaquiline and linezolid. Furthermore, it has been shown that TB DNA can persist longer than 12 weeks in a patient and so we tested sputa collected throughout treatment (either 6 months or 24 months depending treatment arm)[64].

There has been studies that have made use of the MPN assay to differentially culture TB bacteria from patients but never from patients with DR-TB nor patients receiving the new regimen of MDR-TB treatment[58]. The MPN assay will also be used on samples collected from the LCASS, which has not been done previously.

The validity of the CASS system has been shown in previous studies[80,82] (including a submitted manuscript for which I am co-author, Appendix III) and so it is an accurate reference standard against which to measure the new tools we developed to measure infectiousness. Previously,

the SKC BioSamplers have been used to measure other bacteria as well as the influenza virus but has never been used to measure *M. tuberculosis* in cough droplets from patients with TB[86-88,90]. Furthermore, our design for the LCASS is unique and was manufactured for our use.

There has been previous data published on the use of face mask for aerosol testing. This was tested both on patients with COPD as well as patients with TB and was tested on patients recruited from Leicester and The Gambia as well as Pretoria, South Africa[92-94]. However, these were small cohorts and did not look at the effect treatment has on infectiousness, neither was it compared to the only validated tools for measuring infectiousness, CASS. Furthermore, a study was recently published outlining the development of a Respiratory Aerosol Sampling Chamber (RASC) in which a patients sits in an isolated room and various measurements are taken including sampling their aerosol. While this study provided important data, it only looked at pre-treatment patients and not how infectiousness changes on treatment, nor did the make use of gMask or LCASS sampling[98].

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Chapter 2

Mycobacterial genomic DNA from used Xpert MTB/RIF cartridges can be utilised for accurate second-line genotypic drug susceptibility testing and spoligotyping

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Mycobacterial genomic DNA from used Xpert MTB/RIF cartridges can be utilised for accurate second-line genotypic drug susceptibility testing and spoligotyping

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Xpert MTB/RIF (Xpert) is a widely-used test for tuberculosis (TB) and rifampicin-resistance. Second-line drug susceptibility testing (DST), which is recommended by policymakers, typically requires additional specimen collection that delays effective treatment initiation. We examined whether cartridge extract (CE) from used Xpert TB-positive cartridges was, without downstream DNA extraction or purification, suitable for both genotypic DST (MTBDR*plus*, MTBDR*sl*), which may permit patients to rapidly receive a XDR-TB diagnosis from a single specimen, and spoligotyping, which could facilitate routine genotyping. To determine the limit-of-detection and diagnostic accuracy, CEs from dilution series of drug-susceptible and -resistant bacilli were tested (MTBDR*plus*, MTBDR*sl*). Xpert TB-positive patient sputa CEs (n = 85) were tested (56 Xpert-rifampicin-susceptible, MTBDR*plus* and MTBDR*sl*; 29 Xpert-rifampicin-resistant, MTBDR*sl*). Spoligotyping was done on CEs from dilution series and patient sputa (n = 10). MTBDR*plus* had high non-valid result rates. MTBDR*sl* on CEs from dilutions $\geq 10^3$CFU/ml ($C_T \leq 24$, >"low" Xpert semiquantitation category) was accurate, had low indeterminate rates and, on CE from sputa, highly concordant with MTBDR*sl* isolate results. CE spoligotyping results from dilutions $\geq 10^3$CFU/ml and sputa were correct. MTBDR*sl* and spoligotyping on CE are thus highly feasible. These findings reduce the need for additional specimen collection and culture, for which capacity is limited in high-burden countries, and have implications for diagnostic laboratories and TB molecular epidemiology.

Of the 10.4 million individuals with active tuberculosis (TB) in 2015, 580 000 were rifampicin (RIF) resistant or multidrug-resistant (MDR), defined as resistance to isoniazid (INH) and RIF[1]. Only ~20% of MDR-TB cases were diagnosed and started on treatment, and only half started on treatment were cured[1]. Extensively drug-resistant (XDR)-TB, which is MDR with resistance to a fluoroquinolone (FQ) and a second-line injectable drug (SLID) comprises 10% of MDR-TB cases, yet is even more underdiagnosed than MDR-TB, very costly to treat, and represents an emerging public health emergency[2–6].

Xpert MTB/RIF (Xpert) (Cepheid, United States) is a Food and Drug Administration and World Health Organization (WHO)-endorsed nucleic acid amplification test (NAAT) that rapidly detects *Mycobacterium tuberculosis* complex-DNA and RIF-resistance directly from sputa[7–9]. Over 25 million Xpert MTB/RIF cartridges have been consumed and over 30 000 test modules are installed worldwide[10]. The WHO and several national programmes recommend that if Xpert detects resistance, an additional sputum is collected for further drug

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susceptibility testing (DST) using line probe assays (LPAs), such as MTBDR*plus* (RIF and INH) and MTBDR*sl* (FQs and SLIDs), or phenotypic testing[1,9,11,12].

Patients, however, often do not rapidly return to the clinic to give another sputum or receive DST results. For example, a study in South Africa found that, even after MTBDR*plus* roll-out, time-to-treatment since initial diagnosis was unacceptably long (~55 days), and that this was partly due to challenges with patient loss-to-follow-up[13]. Furthermore, many patients do not produce sufficient sputum of adequate quality, especially in settings with high rates of HIV[14–17].

MTBDR*plus* and MTBDR*sl* have suboptimal sensitivity on specimens, and culture is often required prior to DNA extraction and further genotypic testing. Not only can this cause diagnostic delay, but many high burden countries lack the necessary biosafety and laboratory infrastructure for mycobacterial culture and DNA extraction[18–21]. Furthermore, culture can result in the loss of potentially clinically-meaningful resistance[22]. There is hence a need to reduce delays in the diagnosis of drug-resistant TB and use rapid methods that minimise reliance on culture through the direct testing of specimens[23].

Poor adherence to diagnostic algorithms using MTBDR*plus* and MTBDR*sl* has been reported[5,24,25]. For example, in South Africa, 34% of Xpert RIF-resistant patients failed to receive MTBDR*plus* and, of those confirmed to have MDR-TB, 28% did not receive second-line DST with MTBDR*sl* – despite both LPAs being mandated by the national programme[21]. Novel approaches to reduce this gap in the TB care cascade, which is worsened by the requirement for extra patient visits and additional specimen collection, is a major research priority[26,27]. If TB-testing and first- and second-line DST were possible on the first available specimen, fewer patients would potentially be lost and patients could be diagnosed earlier. This could result in earlier effective treatment initiation, fewer patient- and health systems-costs, and better long-term clinical outcomes.

We therefore conducted a proof-of-concept evaluation on whether *M. tuberculosis*-complex genomic DNA in the PCR-reaction mix from used Xpert cartridges (cartridge extract; CE) - that would otherwise be discarded - was detectable in an accurate manner using MTBDR*plus* and MTBDR*sl*. The feasibility of genotyping on CE by spoligotyping was also tested as this would potentially be useful for research laboratories and programmes seeking to implement routine strain surveillance. We explored the feasibility of Sanger sequencing on CE, as this may be useful for additional genotypic DST. Critically, we evaluated CE for all tests without additional downstream DNA extraction or purification, as not only would extraction require equipment not readily available in routine diagnostic laboratories in high burden settings, but it would complicate laboratory workflows and reduce the attractiveness of our approach. If the CE approach was feasible, it would mean that many laboratories would already have instrumentation available for mycobacterial genomic DNA extraction in the form of GeneXpert[10] and not need to procure new equipment.

Material and Methods

Ethics statement. Methods and protocols were carried out in accordance with relevant guidelines and regulations. The study was approved by the Health Research Ethics Committee of Stellenbosch University (N09/11/296) and the City of Cape Town (#10570). Permission was granted to use anonymised residual specimens collected as part of routine diagnostic practice and thus informed consent was waived.

Xpert MTB/RIF on dilution series of drug-susceptible- and drug-resistant bacilli. A triplicate ten-fold dilution series was made using phenotypically-confirmed drug-susceptible (DS)-TB, MDR-TB and XDR-TB clinical isolates (0–10^6 CFU/ml) in phosphate buffer (33 mM Na_2HPO_4, 33 mM KH_2PO_4; pH 6.8) with 0.025% Tween80 (Sigma-Aldrich, United States). Colony counts were done by plating on 7H11 Middlebrook agar (BD Biosciences, United States). Dilutions containing bacilli (1 ml aliquots) were tested by Xpert (54 in total: six dilutions ranging from 10^1–10^6 CFU/ml in triplicate for three strains and hence 18 dilutions each for the DS, MDR, and XDR strains) as well as 0 CFU/ml controls in triplicate, according to the manufacturer's instructions[9]. Used cartridges were stored at 4 °C prior to CE extraction within 24 h and freezing of the CE at −20 °C.

Xpert MTB/RIF on clinical specimens. Used Xpert-*TB*-positive cartridges done on sputa from people with symptoms suggestive of TB tested as part of the South African national TB diagnostic algorithm were collected between February 2016 and November 2016 from the National Health Laboratory Services (NHLS), a South African National Accreditation System-accredited, quality-assured diagnostics laboratory in Cape Town, South Africa[11]. Cartridges were stored at 4 °C prior to CE extraction within 5 days. Fifty-six Xpert TB-positive, RIF-susceptible cartridges and 29 Xpert-TB-positve RIF-resistant cartridges were collected. When the NHLS did a MGIT 960 liquid culture on sputum from RIF-resistant patients, we collected the isolate [20/29 (69%) had available isolates]. Isolates were not available from Xpert TB-positive, RIF-susceptible specimens as culture is not routinely done in these patients[11,28].

Recovery of mycobacterial genomic DNA from used Xpert MTB/RIF cartridges. The transparent diamond-shaped reaction chamber on the back of the cartridge was punctured with a sterile fixed-needle insulin syringe (1 ml; 29 G) (Fig. 1) in a biosafety level 2 cabinet. The full CE volume, typically ~15 µl, was withdrawn and stored in sterile, safe-lock micro-centrifuge tubes at −20 °C prior to analysis. Each cartridge and the surrounding surface was wiped down thoroughly with 1% sodium hypochlorite and 70% EtOH before and after extraction and UV sterilization was done after each batch of extraction. Used needles were discarded in a sharps container containing 1% sodium hypochlorite. Before and after each cartridge extraction session, hood surface area was decontaminated with sodium hypochlorite and EtOH and UV sterilised. No DNA extraction or purification steps were done on CE.

Line probe assays on cartridge extract. MTBDR*plus* and MTBDR*sl* (both version 2.0) were done according to the manufacturer's instructions[29,30] except for Xpert TB-positive, RIF-susceptible clinical specimens CE (n = 56), 7.5 µl CE was used as imput volume into MTBDR*plus* and MTBDR*sl*. For the Xpert TB-positive,

Figure 1. Cartridge extract extraction procedure. (**a**) The arrow indicates the diamond-shaped reaction chamber where the PCR amplification takes place and contains cartridge extract with mycobacterial genomic DNA. The needle is placed at the top of the diamond and the film is slowly and carefully pierced. (**b**) The needle is then slowly inserted deeper into the pocket and cartridge extract mix drawn out without piercing the other side.

RIF-resistant clinical specimen CEs (n = 29) and the dilution series, 5 µl (the recommended imput volume) CE was used in order to have enough CE remaining for Sanger sequencing. MTBDR*plus* and MTBDR*sl* results were reported as susceptible or resistant (RIF and INH for MTBDR*plus*; FQ and SLID for MTBDR*sl*), indeterminate [*M. tuberculosis* complex DNA-positive (reported by the test as TUB-positive) but no gene loci control bands] or TUB-band negative. LPA strips were read by two independent, experienced readers blinded to each other's calls and Xpert results (and, for dilution series, the strain used).

Spoligotyping on cartridge extract. Spoligotyping was done as described[31,32] on 2 µl CE from the MDR-TB dilution series. A set of Xpert TB-positive, RIF-susceptible cartridges (n = 10) done on specimens and separate from those used for genotypic DST on CE were collected with paired culture isolates from an ongoing research study. To determine whether the correct spoligotype was obtained from CE, crude DNA extracted through heat inactivation from the corresponding culture isolates was spoligotyped. SITVIT was used to identify strain families[33].

Targeted Sanger sequencing on cartridge extract. For dilution series, PCR clean-up and Sanger sequencing on 5 µl CE was done by the Stellenbosch University Central Analytical Facility using primers overlapping with LPA-binding sites (Supplementary Table 1). The *gyrA* and *rrs* regions in the DS-TB and XDR-TB strains were sequenced.

Data availability. The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Results

Patient characteristics. A summary of the patient demographic and clinical data is in Table 1. For Xpert TB-positive, RIF-susceptible patients the median age (IQR) was 40 (31–49) years and for RIF-resistant specimens was 35 (23–42) years. 37/55 (67%) of RIF-susceptible patients and 12/20 (60%) of RIF-resistant patients were male.

Feasibility and diagnostic accuracy of MTBDR*plus* and MTBDR*sl* on dilution series Xpert TB-positive cartridge extract. Xpert detected *M. tuberculosis*-complex DNA in all dilutions $\geq 10^2$ CFU/ml and correctly identified RIF-susceptibility and -resistance (Fig. 2). MTBDR*plus* showed poor overall sensitivity for *M. tuberculosis*-complex DNA [22% (12/54) TUB-band-positive] in CE from Xpert TB-positive cartridges. MTBDR*plus* had high rates of non-actionable (TUB-band negative or TUB-band positive but indeterminate) and false RIF-heteroresistant results (Figs 2 and 3).

In contrast, MTBDR*sl* on CE had high sensitivity and specificity [87% (47/54) and 100% (9/9) respectively] for *M. tuberculosis*-complex DNA and a limit of detection of 10^3 CFU/ml. Susceptibility and resistance to FQs and SLIDs were correctly detected for all strains $\geq 10^3$ CFU/ml, corresponding to $C_T \leq 24$ (the higher C_T range of the Xpert "low" semiquantitation category) in all but one sample (one replicate of the MDR-TB strain was indeterminate for FQs; Fig. 3). Once non-actionable results were excluded, overall sensitivities and specificities of 87% (13/15) and 96% (25/26) for FQ-resistance and 94% (15/16) and 97% (30/31) for SLID-resistance, respectively were obtained. When the threshold of $\geq 10^3$ CFU/ml ($C_T \leq 24$) was applied, the sensitivity and specificity were both 100% (12/12 and 23/23, respectively) for FQs and for SLIDs (12/12 and 24/24, respectively).

Patient Characteristics	Xpert TB-positive	
	Xpert rifampicin-susceptible (n = 56)	Xpert rifampicin - resistant (n = 29)
Age, median (IQR)	40 (30–49)	35 (23–42; p = 0.086)
Male gender (%)	37/55 (67)*	12/20 (60)*
Smear-positivity (%)	37/50 (74)*	6/16 (38)*
Culture-positivity (%)	Not done	19/21 (90)*
TTP, median (IQR)	N/A	10 (8–20)
Xpert C_T, median IQR	17.9 (16.3–22.1)	20.5 (16.9–24.8)

Table 1. Patient demographic and clinical data. *Missing data: Gender (n = 1 for RIF-susceptible, n = 9 for RIF-resistant); Smear status (n = 6 for Xpert RIF susceptible, n = 13 for Xpert RIF-resistant); Culture results (n = 8 for RIF-resistant results). Abbreviations: Xpert - Xpert MTB/RIF; IQR - interquartile range; TTP - time-to-positivity; C_T - cycle threshold values.

Diagnostic accuracy of MTBDR*plus* and MTBDR*sl* on clinical specimen Xpert TB-positve cartridge extract. *Xpert MTB/RIF rifampicin-susceptible specimens.* As with the dilution series, MTBDR*plus* had high rates of indeterminate and false-resistance results on clinical specimen CE (Table 2). However, most MTBDR*sl* results from Xpert TB-positive, RIF-susceptible clinical specimen CE were valid (TUB-positive, not indeterminate, and no false-susceptible or -resistant results): 53/56 (95%) for FQ (two TUB-band negative, one indeterminate) and 51/56 (91%) for SLID (two TUB-band negative, three indeterminate). The few CEs that yielded indeterminate MTBDR*sl* results corresponded to "low" or "very low" Xpert semiquantitation levels ($C_T > 24$). The median (IQR) C_T of indeterminate (26.3, 24.4–26.7) vs. determinate (17.62, 15.6–20.6) MTBDR*sl* results differed ($p < 0.001$), indicating that indeterminate results are likely a function of low DNA concentrations in CE. There was not enough CE volume remaining or a matching clinical isolate for confirmatory testing from the three MTBDR*sl*-detected SLIDs resistant patients.

Xpert MTB/RIF rifampicin-resistant specimens. MTBDR*sl* on Xpert TB-positive, RIF-resistant CE had 24/29 (83%) valid results. For FQs, 14/24 (58%) were susceptible and 10/24 (42%) were resistant. For SLIDs, 15/24 (63%) were susceptible and 9/24 (37%) resistant. The five non-valid results were TUB-band-negative [2/29 (7%)] or indeterminate for both FQs and SLIDs [3/29 (10%); Table 2]. All CEs corresponding to the higher C_T ranges of the Xpert "low" semiquantitation category ($C_T \leq 24$) had valid results, whereas those that had indeterminate or TUB band-negative results corresponded to the lower semiquantitation levels ($C_T > 25.0$). The median (IQR) C_T of indeterminate (29.1, 26.5-31.1) vs. determinate (20.5, 16.−23.2) results differed significantly ($p < 0.001$).

MTBDRplus and MTBDRsl performance on Xpert MTB/RIF cartridge extract by smear status. MTBDR*plus* had high non-valid result rates irrespective of smear status. However, MTBDR*sl* on CE from smear-negative sputums had significantly higher rates of non-actionable results [5/23 (22%) vs. 1/43 (2%) for FQ, p = 0.01; 6/23 (23%) vs. 2/43 (5%) for SLIDs, p = 0.01] compared to smear-positive patients (Supplementary Table 2).

Concordance of MTBDRsl results on cartridge extract and culture isolates. Of the 29 Xpert TB-positive, RIF-resistant patients, 20 (69%) matched culture isolates were collected while the remaining nine had negative or contaminated cultures. The CEs and isolates showed 18/20 (90%) matching MTBDR*sl* FQ results and 17/20 (84%) matching SLID results. There were 2/20 (10%) discordant TUB-band MTBDR*sl* results on culture isolates (one TUB-positive and FQ and SLID sensitive, one TUB-positive and FQ and SLID resistant) where both CE results were TUB-band negative. There was also 1/20 (5%) discordant SLID result (CE showed resistance but the isolate showed susceptibility). Importantly, all three discordant results corresponded to a "very low" semiquantitation ($C_T > 28.0$). All TUB-band, susceptibility and resistance calls were concordant at $C_T \leq 24$, indicating that the diagnostic accuracy of MTBDR*sl* on CE vs. isolates is likely comparable at this threshold.

Spoligotyping on cartridge extract. *Dilution series.* Spoligotyping resulted in a readable strain type for dilutions $\geq 10^3$ CFU/ml, corresponding to the same threshold seen for MTBDR*sl*.

Clinical specimens. Spoligotyping on specimen CE and crude DNA from matched culture isolates were highly concordant 10/10 (100%) at the threshold defined by the dilution series (Table 3). A variety of strain families were observed with Beijing as the predominant family type [6/10 (60%)] as well as 2/10 (20%) LAM and 2/10 (20%) T1 family type.

Targeted sequencing on extract from used Xpert MTB/RIF cartridges. *Dilution Series.* Targeted Sanger sequencing was done on dilution series CE. For the *rrs* PCR on CE, sequence shorter than the expected length was observed. PCR of *gyrA* from CE from dilutions 10^3–10^4 CFU/ml resulted in sequence expected length, however high background noise occurred and the sequence did not align to H37Rv [NC_000962]. *gyrA* on CE from dilutions 10^5–10^6 CFU/ml aligned to the reference genome, however, several SNPs known to be present in the resistance determining regions (identified by sequencing of the corresponding isolate) were not detected. Due to the relatively poor limit of detection and accuracy of Sanger sequencing on dilution series CE, we did not do sequencing on clinical specimen CEs.

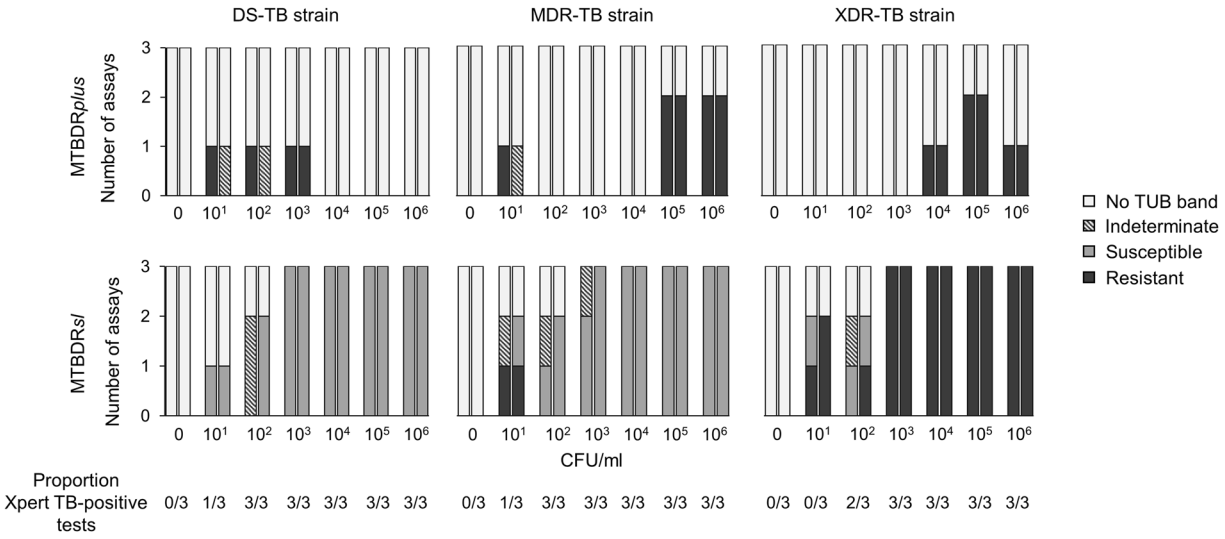


Figure 2. Results of MTBDR*plus* and MTBDR*sl* on Xpert CE from a dilution series of DS-, MDR- and XDR-TB strains. MTBDR*plus* (irrespective of concentration and strain) had high TUB-band negativity and indeterminate rates. However, MTBDR*sl* had high sensitivity and specificity and low indeterminate rates. For each dilution, left bars are for rifampicin (MTBDR*plus*, top row) or fluoroquinolones (MTBDR*sl*, bottom row) and right bars are for isoniazid (MTBDR*plus*) or second-line injectables (MTBDR*sl*). Data from LPA on DS-TB, MDR-TB and XDR-TB strains are shown. The experiment was done in triplicate. Abbreviations: CFU – colony forming; DS-TB – drug susceptible TB; MDR-TB – multidrug resistant TB; XDR-TB – extensively drug resistant TB; units; Xpert - Xpert MTB/RIF.

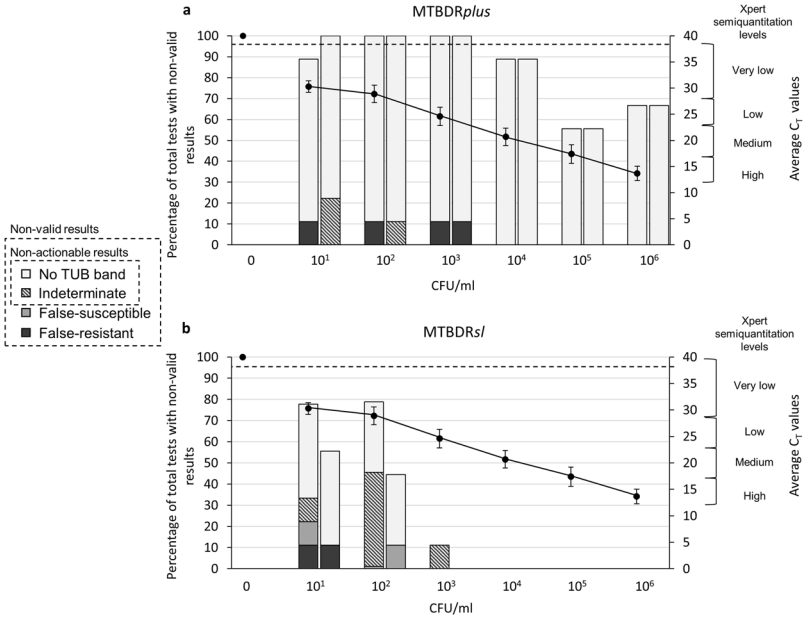


Figure 3. Xpert MTB/RIF quantitative information [average cycle threshold (C_T) values] (line graph, right y-axes) versus bacterial load (CFU/ml) in a triplicate dilution series for MTBDR*plus* (**a**) and MTBDR*sl* (**b**) done on CE. Left y-axes (bars) show the proportion of assays with non-valid results, disaggregated into non-actionable (TUB-band negative, indeterminate) and non-valid (false-susceptible, false-resistant). For each dilution, left bars are for rifampicin (MTBDR*plus*, top) or fluoroquinolones (MTBDR*sl*, bottom) and right bars are for isoniazid (MTBDR*plus*) or second-line injectables (MTBDR*sl*). Beyond 10^3 CFU/ml, there were no false resistance or susceptibility calls for MTBDR*sl*, which corresponds to $C_T \leq 24$. $C_T \geq 38$ (horizontal dashed line) correspond to a negative Xpert. Error bars show standard error (SE) of average C_T. Right y-axes show C_T corresponding to Xpert semiquantitation levels of very low ($C_T > 28$), low ($C_T = 22–28$), medium ($C_T = 16–22$) and high ($C_T < 16$). Pooled data from LPAs on DS-TB, MDR-TB and XDR-TB strains are shown. Abbreviations: CFU – colony forming; DS-TB – drug susceptible TB; MDR-TB – multidrug resistant TB; XDR-TB – extensively drug resistant TB; CFU – colony forming units; Xpert - Xpert MTB/RIF.

All Xpert TB-positive specimens						Xpert TB-positive specimens with $C_T \leq 24$					
Xpert rifampicin-susceptible				Xpert rifampicin-resistant		Xpert rifampicin-susceptible				Xpert rifampicin-resistant	
MTBDR*plus* (n = 56)		MTBDR*sl* (n = 56)		MTBDR*sl* (n = 29)*		MTBDR*plus* (n = 49)		MTBDR*sl* (n = 49)		MTBDR*sl* (n = 20)*	
TUB-band positive (%) 47/56 (84)		**TUB-band positive (%) 47/54 (96)**		**TUB-band positive (%) 27/29 (93)**		**TUB-band positive (%) 45/49 (92)**		**TUB-band positive (%) 49/49 (100)**		**TUB-band positive (%) 20/20 (100)**	
Rifampicin (%)		**Fluoroquinolones (%)**		**Fluoroquinolones (%)**		**Rifampicin (%)**		**Fluoroquinolones (%)**		**Fluoroquinolones (%)**	
Susceptible	0/47 (0)	Susceptible	53/54 (98)	Susceptible	14/27 (52)	Susceptible	0/56 (0)	Susceptible	49/49 (100)	Susceptible	11/20 (55)
Resistant	47/47 (100)	Resistant	0/54 (0)	Resistant	10/27 (37)	Resistant	45/49 (92)	Resistant	0/49 (0)	Resistant	9/20 (45)
Indeterminate	0/47 (0)	Indeterminate	1/54 (2)	Indeterminate	3/27 (11)	Indeterminate	0/49 (0)	Indeterminate	0/49 (0)	Indeterminate	0/20 (0)
Isoniazid (%)		**Second-line injectables %)**		**Second-line injectables (%)**		**Isoniazid (%)**		**Second-line injectables (%)**		**Second-line injectables (%)**	
Susceptible	11/47 (23)	Susceptible	48/54 (88)	Susceptible	15/27 (56)	Susceptible	11/49 (23)	Susceptible	46/49 (94)	Susceptible	14/20 (70)
Resistant	0/47 (0)	Resistant	3/54 (6)	Resistant	9/27 (33)	Resistant	0/49 (0)	Resistant	1/49 (2)	Resistant	6/20 (30)
Indeterminate	36/47 (77)	Indeterminate	3/54 (6)	Indeterminate	3/27 (11)	Indeterminate	34/49 (69)	Indeterminate	2/49 (4)	Indeterminate	0/20 (0)
TUB-band negative (%) 9/56 (16)		**TUB-band negative** (%) 2/56 (4)		**TUB-band negative** (%) 2/29 (7)		**TUB-band negative** (%) 4/49 (8)		**TUB-band negative** (%) 0/49 (0)		**TUB-band negative** (%) 0/20 (0)	

Table 2. Results of MTBDR*plus* and MTBDR*sl* drug susceptibility testing using cartridge extract on clinical specimens. MTBDR*plus* had high indeterminate results and rifampicin-resistance false-positive rates. MTBDR*sl* had low indeterminate rates for both DS-TB and DR-TB specimens and performance improved when MTBDR*sl* was done only on specimens with $C_T \leq 24$. *For the 29 Xpert RIF-resistant specimens we were able to retrieve 20 paired culture isolates used for MTBDRsl. 18/20 matched for FQs and 17/20 for SLIDs, the 2/20 done on crude DNA had LPA results whereas the LPA on CE was TUB-band negative. 1/20 did not match for the SLID resistance. Both the TUB-band negative and discordant SLIDs result corresponded to "very low" semi-quantitation level. When defined threshold of $C_T \leq 24$ was applied all LPAs on CE matched LPA from culture isolates.

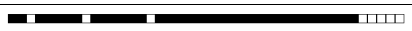
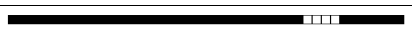
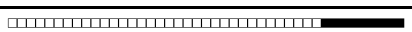
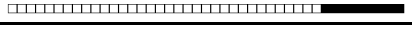
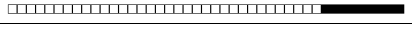
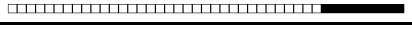
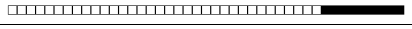
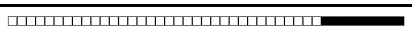
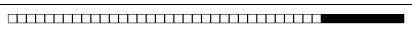
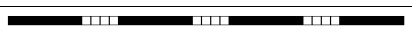
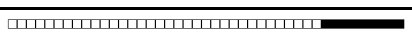
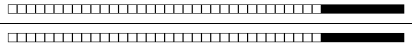
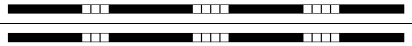
Material used for spoligotyping	Xpert MTB/RIF semi-quantitation level	C_T	Spoligotyping pattern	Family
Negative Control			N/A	N/A
H37Rv				H37Rv
BCG				BOVIS1_BCG
Isolate (a)	-	-		T1
CE (a)	MEDIUM	20.85		T1
Isolate (b)	-	-		BEIJING
CE (b)	HIGH	12.28		BEIJING
Isolate (c)	-	-		BEIJING
CE (c)	HIGH	16.20		BEIJING
Isolate (d)	-	-		BEIJING
CE (d)	HIGH	15.00		BEIJING
Isolate (e)	-	-		BEIJING
CE (e)	LOW	24.40		BEIJING
Isolate (f)	-	-		BEIJING
CE (f)	HIGH	14.74		BEIJING
Isolate (g)	-	-		LAM9
CE (g)	LOW	20.86		LAM9
Isolate (h)	-	-		BEIJING
CE (h)	MEDIUM	21.35		BEIJING
Isolate (i)	-	-		LAM3
CE (i)	HIGH	16.28		LAM3
Isolate (j)	-	-		T1
CE (j)	MEDIUM	21.88		T1

Table 3. Spoligotyping results performed on CE done on sputum specimens and paired culture isolates at defined threshold ($C_T \leq 24$).

Discussion

Our key findings are: (1) MTBDR*sl* on CE enabled genotypic drug-susceptibility testing for FQs and SLIDs with high accuracy and low indeterminate rates when the Xpert semiquantitation category was at least "medium" or $C_T \leq 24$ (corresponding to $\geq 10^3$ CFU/ml), (2) spoligotyping was feasible and accurate on CE at the same threshold, (3) MTBDR*plus* was not feasible or accurate on CE and (4) neither was Sanger sequencing. These data have implications for the routine diagnosis of drug-resistant TB, researchers, and test developers.

Xpert is one of the most widely used tests for TB and drug-resistance[9,34] and although it is a significant advancement, time-to-treatment – especially for MDR- and XDR-TB - is still very long[35–38]. Our results show that accurate second-line drug testing using MTBDR*sl* is possible on CE from Xpert cartridges that would otherwise be discarded. This potentially allows for a rapid, single-specimen diagnosis of XDR-TB without additional specimen collection. Importantly, we defined a threshold at which this approach is feasible, meaning that MTBDR*sl* assays do not need to be wasted on CE unlikely to give a valid result. Using this threshold, we showed that on clinical specimen CEs, susceptibility and resistance calls were concordant with those from the isolate[19,39]. Furthermore, we showed that it is possible to do spoligotyping on CE at this threshold, which will inform strain surveillance and research studies on relapse and reinfection where specimens are limited. Collectively, these findings may reduce the need for culture.

Although our data suggest that the MTBDR*sl* will work on CE from cartridges with an Xpert semiquantitation category of at least "low", we suggest that, in laboratories where C_T cannot be readily calculated, a category of at least "medium" is used to guide use of this strategy unless the laboratory is comfortable with some semiquantitation low specimens not having a valid MTBDR*sl* result. Alternatively, if smear microscopy is available, smear-positivity may be used to guide use of CE, however, some smear-negative specimens in whom this approach would work (10^3-10^4 CFU/ml) would be unnecessarily excluded.

When considering the CE approach, it is important to identify a safe and sterile environment to avoid contamination. Although Xpert sample reagent as well as the sonication lysis step within the cartridge helps ensure *M. tuberculosis* is no longer culturable (and therefore poses minimal infectious risk[40]), steps to minimise the risk of *rpoB* amplicon cross-contamination should be implemented. These can include working in a dedicated cabinet or room and sterilising the work area with UV and disinfectant after CE is collected. Importantly, however, cross-contamination of other Xpert cartridges with *rpoB* amplicons appears unlikely. Although Xpert's automated pre-amplification wash step does not remove large pieces of debris-associated genomic DNA, it does efficiently remove high concentrations of contaminating *rpoB* amplicons from assays like MTBDR*plus*[41,42]. NAATs without such a wash step may be more vulnerable to CE *rpoB* amplicon cross-contamination.

Our study differed from a previous study which showed that sequencing, MTBDR*plus*, spoligotyping and MIRU-VNTR typing are feasible on the sputum mixed with Xpert sample reagent[43]. However, this sample reagent method has a number of disadvantages: 1) often no volume remains, 2) prolonged exposure to sample reagent degrades DNA and potentially introduces mutations[9,40], and 3) it still requires DNA extraction prior to PCR. Furthermore, DNA extraction adds cost and is not always feasible in laboratories in high burden countries; whereas the CE method yields directly usable material and does not need additional extraction or purification steps. An advantage, however, of using the sputum mixed with Xpert reagent buffer, is that it likely avoids high MTBDR*plus* error rates (TUB-band negative, indeterminate, false-positive) seen with CE. This could be due to the large amount of *rpoB* amplicons in Xpert TB-positive CE, which share binding sites with MTBDR*plus* probes and confound the assay resulting in non-valid results. Furthermore, the *rpoB* PCR that occurs as part of MTBDR*plus* may sequester reagents away from the multiplex *inhA* and *katG* amplification reactions. Testing for mutations conferring INH resistance using CE might hence be possible with the Genoscholar INH II line probe assay (which does not contain *rpoB* probes)[44]. Sequencing from CE thus primarily appears to be driven by *rpoB* amplicon interference (although a PCR clean-up was done prior to sequencing, this would have co-purified *rpoB* amplicons). Further investigation with primers optimised for minimal-input DNA may be warranted, however, it appears that, for sequencing, the best approach to avoid contaminating amplicons might be to PCR from the specimen-Xpert sample reagent mixture[43]. Given the rates of non-valid CE results below the defined threshold, we suggest that specimen-Xpert sample reagent mix be kept in the event that C_T falls >24.

The results presented here should be interpreted in context of their limitations. For the clinical specimens tested from the NHLS, matched culture isolates were not available for Xpert RIF-susceptible specimens, as per the national algorithm. However, the dilution series experiments showed very high concordance between MTBDR*sl* on CE vs. the isolates. The utility of CE depends on the downstream test used and MTBDR*sl* susceptible or non-valid results should be interpreted from CE the same as when they are done on patient specimens (i.e., further investigation, including culture, is recommended)[45]. Realistically, cartridges may need to be transported from remote locations and so the effect of storing cartridges for prolonged duration (>5 days) and at ambient temperature requires further systematic testing. Using bacilli in buffer can have limitations, which is why we also used patient clinical specimens, which are a better material to test than bacilli added to sputum (the former has bacilli within a sputum matrix, whereas in the latter bacilli are typically freely floating in bubbles).

In conclusion, CE contains template DNA for second-line DST using MTBDR*sl*, resulting in accurate results highly concordant with those from isolates, provided bacillary load in the specimen corresponds to at least a "medium" Xpert semiquantitation category of $C_T \leq 24$. This potentially facilitates XDR-TB detection within days from a single specimen. Spoligotyping is also feasible on CE and works consistently at this threshold. Our method provides an opportunity to potentially reduce the burden associated with addition specimen collection, such as patient treatment delay, pre-treatment loss-to-follow-up, and increased patient and provider costs. Furthermore, it shows that material that would otherwise be discarded still holds diagnostic utility.

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Author Contributions

G.T., R.W. and M.D.V. conceived the experiments. R.V., B.D., S.P., N.K. and A.R. conducted the experiments. J.S. and T.D. provided specimens and data from the NHLS. R.V. and B.D. analysed the data. All authors reviewed the manuscript.

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Chapter 3

Extract from used Xpert MTB/RIF Ultra cartridges is useful for accurate second-line drug-resistant tuberculosis diagnosis with minimal *rpoB*-amplicon cross contamination risk

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Extract from used Xpert MTB/RIF Ultra cartridges is useful for accurate second-line drug-resistant tuberculosis diagnosis with minimal *rpoB*-amplicon cross-contamination risk

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Xpert MTB/RIF Ultra (Ultra) detects *Mycobacterium tuberculosis* and rifampicin resistance. Follow-on drug susceptibility testing (DST) requires additional sputum. Extract from the diamond-shaped chamber of the cartridge (dCE) of Ultra's predecessor, Xpert MTB/RIF (Xpert), is useful for MTBDR*sl*-based DST but this is unexplored with Ultra. Furthermore, whether CE from non-diamond compartments is useful, the performance of FluoroType MTBDR (FT) on CE, and *rpoB* cross-contamination risk associated with the extraction procedure are unknown. We tested MTBDR*sl*, MTBDR*plus*, and FT on CEs from chambers from cartridges (Ultra, Xpert) tested on bacilli dilution series. MTBDR*sl* on Ultra dCE on TB-positive sputa (n = 40) was also evaluated and, separately, *rpoB* amplicon cross-contamination risk . MTBDR*sl* on Ultra dCE from dilutions $\geq 10^3$ CFU/ml (C_{Tmin} <25, >"low semi-quantitation") detected fluoroquinolone (FQ) and second-line injectable (SLID) susceptibility and resistance correctly (some SLIDs-indeterminate). At the same threshold (at which ~85% of Ultra-positives in our setting would be eligible), 35/35 (100%) FQ and 34/35 (97%) SLID results from Ultra dCE were concordant with sputa results. Tests on other chambers were unfeasible. No tubes open during 20 batched extractions had FT-detected *rpoB* cross-contamination. False-positive Ultra *rpoB* results was observed when dCE dilutions $\leq 10^{-3}$ were re-tested. MTBDR*sl* on Ultra dCE is concordant with isolate results. *rpoB* amplicon cross-contamination is unlikely. These data mitigate additional specimen collection for second-line DST and cross-contamination concerns.

Drug-resistant tuberculosis (TB) remains a global threat[1]. Of 10 million estimated incidence cases reported in 2017, 588 000 were rifampicin-resistant[2]. Of these ~458 000 were multidrug-resistant (MDR). Despite the improved roll-out of rifampicin-resistance testing, many patients are not diagnosed appropriately or started on effective treatment, resulting in huge TB care cascade gaps[3,4]. For example, in South Africa, 84% of patients with drug-resistant TB have access to rifampicin-susceptibility testing, but only 47% of these are started on likely effective treatment[4]. Similarly, in India, only 41% of the MDR-TB burden was diagnosed in 2013 and, of these, just 32% started on treatment[5]. Innovative approaches are needed to ensure more patients receive comprehensive drug susceptibility testing (DST).

Previous work showed that mycobacterial genomic DNA can be recovered from the rear diamond-shaped chamber of used Xpert MTB/RIF (Xpert) cartridges after the test is complete. This diamond cartridge extract (dCE) is useful for downstream testing with the MTBDR*sl* line probe assay (LPA) (Hain Lifescience, Germany), the only World Health Organization (WHO)-endorsed molecular test for second-line drug resistance, and

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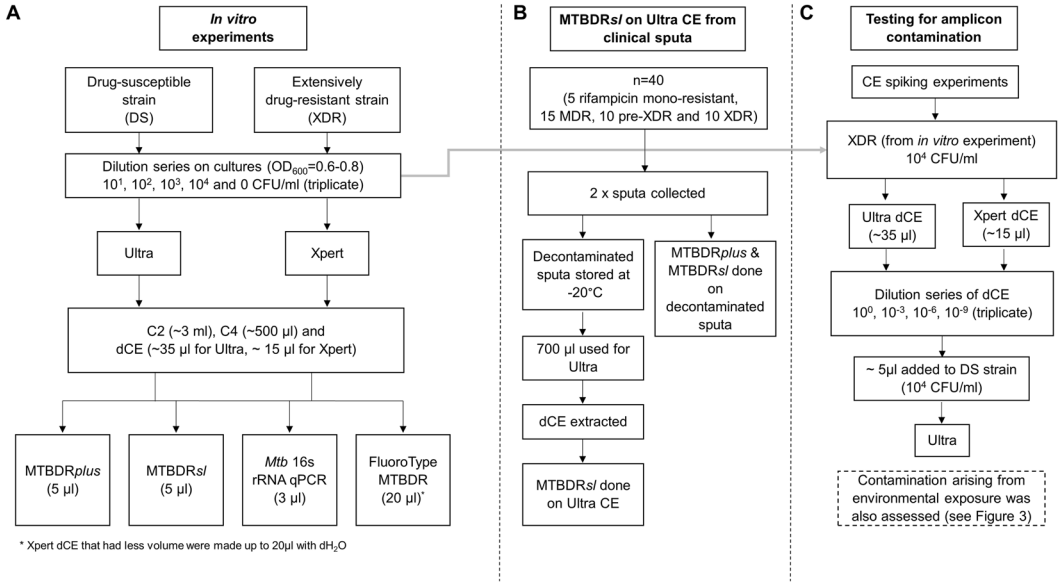

Figure 1. Study flow diagrams for the (**A**) *in vitro* experiment, (**B**) MTBDR*sl* on Ultra CE from clinical sputa experiment, and the (**C**) evaluation of *rpoB* amplicon cross-contamination risk experiment.

spoligotyping[6], a method useful for monitoring the molecular epidemiology of TB outbreaks. This additional testing does not require extra specimen collection nor additional downstream DNA extraction, both of which can exacerbate patient loss within the diagnostic care cascade.

As Xpert is a real-time PCR that generates quantitative information, a cycle threshold value (C_T <24) was identified at which downstream dCE testing using MTBDR*sl* was successful and fully concordant with MTBDR*sl* results on matching isolates[7]. However, Xpert dCE was not useful for first-line DST using the WHO-endorsed MTBDR*plus* assay, likely due to interference from large numbers of Xpert *rpoB* amplicons. In addition to the dCE approach, others[8,9] have shown it is possible to test leftover specimen-sample reagent mix remaining after Xpert, however, remnant volume is not always present and DNA extraction and downstream clean-up might still be needed.

Xpert MTB/RIF Ultra (Ultra) recently superseded Xpert as WHO-endorsed frontline molecular test-of-choice for TB and rifampicin resistance[10]. Compared to Xpert, Ultra has higher sensitivity in paucibacillary samples, however, specificity is overall lower[11–13]. Ultra is a different assay compared to Xpert and it is not necessarily given that the extract approach would be feasible on Ultra dCE. We aimed to confirm that Ultra dCE would be useful for second-line DST. Furthermore, we asked if extract from other chambers within the cartridge other than the diamond (i.e., chambers that are likely *rpoB* amplicon-free), may contain DNA. We quantified this DNA using a *Mycobacterium tuberculosis* complex 16S rRNA real time qPCR and evaluated whether this DNA was useful for first-line DST using the FluoroType MTBDR (FT) (Hain Lifescience, Germany) assay[14,15]. A test such as FT could, for example, be used to check for isoniazid mono-resistance or confirm Ultra rifampicin-resistance results.

Lastly, as the cartridge extraction (CE) procedure involves aspirating fluid rich in *rpoB* amplicons, it may represent a source of cross-contamination. We sought to evaluate this risk, both under a prolonged exposure scenario (where collection tubes were purposely exposed during extended batch extractions) and an absolute worst-case scenario (directly adding dCE to a sample later tested by Ultra). Showing that the extracted cartridge approach in Ultra is compatible with MTBDR*sl* and represents minimal *rpoB* amplicon cross-contamination risk would increase the likelihood of implementation, especially as Xpert is in the process of being phased out in lieu of Ultra. In turn, this could reduce both sputum collection requirements for complete DST and time-to-effective-treatment initiation.

Methods

Ethics statement. Methods and protocols were carried out in accordance with relevant guidelines and regulations. The study was approved by the Health Research Ethics Committee of Stellenbosch University (N09/11/296) and the City of Cape Town (10570). Permission was granted to use anonymised residual specimens collected during routine diagnostic practice and thus patient informed consent was waived.

Ultra and Xpert on dilution series of *Mycobacterium tuberculosis* strains. Culturing of genotypically-confirmed drug-susceptible (DS-TB) and extensively-drug resistant (XDR) *M. tuberculosis* isolates were done in a Biosafety Level (BSL) 3 laboratory to an OD_{600} of 0.6–0.8 (Fig. 1A). A triplicate tenfold dilution series from three separate cultures [10^0–10^4 colony forming units (CFU)/ml] was prepared in phosphate buffer (33 mM Na_2HPO_4, 33 mM KH_2PO_4; pH 6.8) with 0.025% Tween80 (Sigma-Aldrich, United States). Colony counts were done on 7H11 Middlebrook agar (BD Biosciences, United States). A total of 52 dilutions [four dilutions, 10^1–10^4 CFU/ml in triplicate for both strains plus a negative control for each strain; $(4 \times 3 \times 2 + 2) \times 2$] were made up to 1 ml and tested by Ultra (n = 26) or Xpert (n = 26) per the manufacturer's instructions[16,17]. Used positive cartridges were stored prior to extraction at 4 °C for ≤3 days. Crude DNA (heat inactivated for

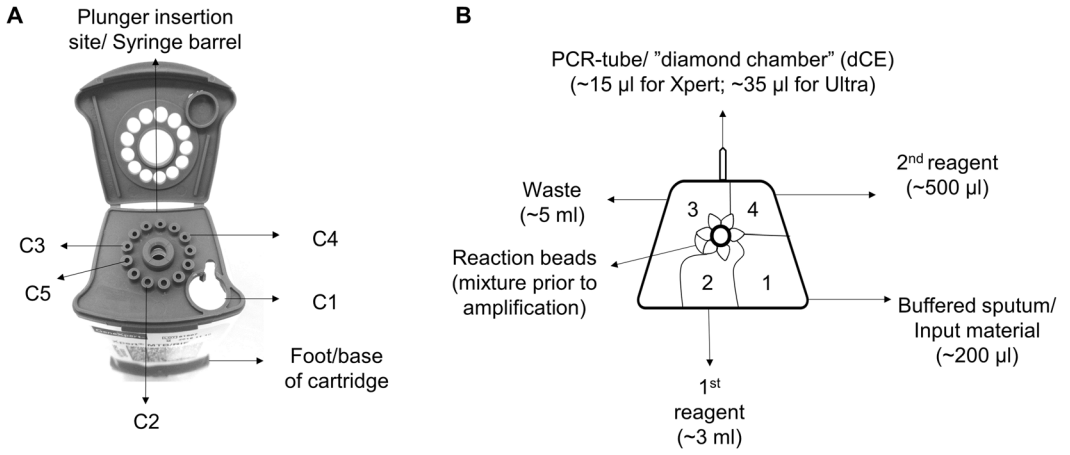

Figure 2. (**A**) Entry points through the lid of the cartridge for access to different cartridge chambers. (**B**) Top-down cross-section of the inside of the cartridge corresponding to the access points.

2 hours at 100 °C) from the same strains served as positive controls for downstream tests (16S rRNA gene qPCR, MTBDR*plus*, MTBDR*sl*, FT).

Ultra on sputum from TB patients. Forty used positive Ultra cartridges done on NALC-NaOH decontaminated sputa from pre-treatment TB patients with known drug resistance [5 rifampicin-mono-resistant, 15 MDR, 10 pre-XDR (resistance to rifampicin, isoniazid and either a fluoroquinolones or a second-line injectable), 10 XDR] were collected from November 2015 to September 2017 and dCEs were extracted as described previously[6] (Fig. 1B). To confirm MTBDR*sl* results from dCEs, MTBDR*sl* was done per the manufacturer's instructions directly on corresponding decontaminated sputa[18,19]. Ultra cartridges were processed in a manner blinded to MTBDR*sl* results.

Recovery of mycobacterial genomic DNA from used Ultra and Xpert cartridges. *Preparation of work space.* BSL2 hood surfaces were sterilised [1% NaOCl (bleach), 70% EtOH, 5 min UV irradiation] before and after each batched extraction. Each cartridge was wiped with 1% bleach and 70% EtOH before and after each extraction.

Description of cartridge design. To investigate the feasibility of testing extract from Ultra and Xpert cartridge chambers, an understanding of their design and inner processes is required. As described previously, each cartridge has a similar design consisting of a foot, valve, body, reaction tube and lid[20,21]. The five internal chambers hold buffers and lyophilised PCR reagents used for sputum homogenisation, washing away debris, and DNA extraction, purification, and amplification[22]. The Xpert and Ultra procedures, including the processes inside the cartridges and the contents of each chamber are described in the supplement. After assay conclusion, the volumes typically remaining in each chamber are ~500 µl for Chamber 1 (C1), ~3 ml for Chamber 2 (C2), ~5 ml for Chamber 3 (C3) and ~500 µl for Chamber 4 (C4) [Chamber 5 (C5) had no volume remaining after test completion].

Diamond chamber extract. dCEs were extracted from all positive cartridges by puncturing the rear chamber with a sterile 29 G × 1/2″ 1 ml insulin syringe (Avacare, South Africa) (Fig. 2A,B) as described previously[6]. The full volume was extracted (~15 µl for Xpert; ~35 µl for Ultra). CEs were stored in microcentrifuge tubes at −20 °C prior to analysis.

Other chambers. Five cartridge chambers (C1, C2, C3, C4, C5) were accessed by inserting a 22 G spinal needle (Becton Dickinson, United States) fixed a 5 ml syringe (Fig. 2A; a pipette may also be used for C1) and the entire volume withdrawn (Fig. 2A,B). C5 had no remaining volume left after Xpert or Ultra test completion. No DNA extraction or purification steps were done for downstream assays.

16s rRNA gene quantitative PCR (qPCR) on cartridge extract. CEs from C1–4 and dCE from Ultra and Xpert done on the serial dilutions were tested (heat extracted crude DNA from matching isolates was used as positive control). For each qPCR, 5 µl iTaq Universal SYBR Green Supermix (Bio-Rad), 0.3 µl (300 nM) of *M. tuberculosis* specific forward (V4 515F) primers, 0.3 µl (300 nM) of *M. tuberculosis* specific reverse (V4 806R) primers (Table S1) and 1.4 µl nuclease-free water was used[23]. 3 µl CE was added and amplification occurred using a Bio-Rad CFX-96. The threshold used to determine if a reaction was excluded from subsequent analyses was defined as a C_q value greater than the average of the triplicate negative controls for that run. Chambers with a C_q less than that average value were considered positive for *M. tuberculosis* complex (MTBC) DNA and used for MTBDR*plus*, MTBDR*sl* and FT.

MTBDR*plus* and MTBDR*sl* line probe assays on cartridge extract. *Diamond chamber extract.* MTBDR*plus* and MTBDR*sl* (both version 2.0) were performed on dCEs from Ultra and Xpert done on the *in vitro* dilution series. For Ultra done on sputa from patients, only MTBDR*sl* was done. 5 µl dCE was used for MTBDR*plus* and MTBDR*sl* each. MTBDR*plus* and MTBDR*sl* results were reported as described[24]: either actionable [TUB-band positive and determinate (gene-specific locus bands present)] or non-actionable [TUB-band negative or TUB-band positive but indeterminate (gene-specific locus band absent)]. Susceptibility calls were made for all actionable results. Banding patterns were read by two experienced independent readers blinded to each other's calls, the Ultra and Xpert results, and, for the dilution series experiement, the strain antibiograms (if there was a discrepancy between readers, a third experienced reader reviewed results and did the final classification).

Other chambers. MTBDR*plus* and MTBDR*sl* were done on C2 and C4 CEs from both Ultra and Xpert done on the dilution series. C1, C3, and C5 were not tested with LPAs as their CEs were 16S rRNA qPCR-negative or there was no volume remaining to test after the Ultra or Xpert test had completed (C5).

FluoroType MTBDR on cartridge extract. *Diamond chamber.* dCEs from Ultra and Xpert cartridges done on the *in vitro* dilution series were tested by FT using the manufacturer's instructions[25]. A total of 26 tubes for each test (Ultra, Xpert) were tested [four dilutions from 10^1–10^4 CFU/ml in triplicate for both strains plus a negative control for each strain, $(4 \times 3 \times 2 + 2)$]. As Xpert dCE had a volume of ~15 µl, after MTBDR*plus* (5 µl), MTBDR*sl* (5 µl), and the 16S rRNA qPCR (3 µl) were all done on the same Xpert dCE, the remaining volumes (5–14 µl) were made up to 20 µl with dH_2O for FT (the recommended input volume)[25]. All Ultra dCEs (~35 µl originally) had 20 µl remaining and the full 20 µl was used for FT. FT results were classified in a manner similar to that for the line probe assays: actionable (MTBC detected; rifampicin and isoniazid susceptible or resistant) or non-actionable (no MTBC detected, MTBC indeterminate or MTBC detected but rifampicin or isoniazid indeterminate).

Other chambers. FT was done on C2 and C4 (as for LPAs) from both Ultra and Xpert cartridges used for the dilution series.

Evaluation of *rpoB* amplicon cross-contamination risk. *Amplicon escape during batched cartridge extractions.* During all Ultra and Xpert diamond chamber extractions, 1.5 ml microcentrifuge tubes containing 100 µl sterile dH_2O were positioned in the same BSL2 cabinet (Fig. 3A). Three tubes remained open throughout all extractions for each batch extraction and three remained closed (negative controls). Tubes were stored at −20 °C for later FT testing. A total of 20 batches of cartridges were extracted [n = 120 tubes in total from the 20 batches, n = 60 open tubes and n = 60 closed tubes including triplicates], with a median (IQR) number of cartridges per batch of 17.5 (10.5–27.5). There were also three tubes open for each individual cartridge extraction but these were not tested further based on results of the open tubes during batched extraction, which revealed no cross-contamination. Furthermore, extractions procedures were done by a total of five different users to reflect user variability.

Spiking of amplicons. The same XDR-TB strain with known Xpert and Ultra *rpoB* resistance profiles was used in the dilution series (Fig. 1C). Ultra and Xpert were each done on 1 ml of a 10^4 CFU/ml concentration (in triplicate). dCEs were extracted and used for a dilution series (10^0, 10^{-3}, 10^{-6}, and 10^{-9}; each 1 ml final volume). For all dilutions, 5 µl was added to 700 µl of the DS-TB strain (10^4 CFU/ml) and tested with Ultra [700 µl was used as, when combined with the recommended two-fold sample reagent volume, the 2 ml input volume is reached with minimal sample unused (~100 µl)].

Results

***Mycobacterium tuberculosis* complex genomic DNA detection in different chambers from cartridges done on dilution series.** Though qPCR-positive results were obtained from C2, C4 and the dCE (Fig. S1), these results were highly variable even at high concentrations of bacilli (at least 10^4 CFU/ml), suggesting interference. As C2, C4 and dCE gave positive qPCR results on cartridges done on some dilutions, and C1 and C3 gave none, we only explored the utility of the former for downstream testing using MTBDR*plus*, MTBDR*sl*, and FT.

MTBDR*plus* and MTBDR*sl* on extract from cartridges done on dilution series. *TB detection.* More Ultras were MTBC-positive at lower CFU titres than Xpert [e.g., 4/6 (67%) of the 10^1 CFU/ml aliquots vs. 1/6 (17%) for Xpert at the same concentration for both strains] (Fig. 4). MTBDR*plus* had high rates of non-actionable results across all dilutions irrespective of the cartridge chamber extract originated from (diamond, C2, C4) or initial test (Ultra, Xpert) (Fig. 4). MTBDR*sl* had actionable results for all Ultra dCEs $\geq 10^3$ CFU/ml and, for Xpert, all but one dCE $\geq 10^3$ CFU/ml (one Xpert replicate at 10^3 CFU/ml was MTBDR*sl*-non-actionable). MTBDR*sl* on C2 and C4 had non-actionable results across all dilutions (Ultra and Xpert).

Resistance detection. MTBDR*sl* correctly identified FQ and SLID resistance on Ultra dCE done on all XDR strain aliquots $\geq 10^3$ CFU/ml (Fig. 5). On the DS-TB strain, MTBDR*sl* identified FQ susceptibility in all three 10^4 CFU/ml replicates and in 2/3 (67%) replicates for SLIDs (one indeterminate). At 10^3 CFU/ml for the DS-TB strain, 2/3 (67%) were correctly identified as FQ susceptible (one indeterminate) and all were SLID-indeterminate. The C_{Tmin} threshold at which all MTBDR*sl* results was feasible on Ultra CE was <25, which was used for further experiments. Similar results were obtained for MTBDR*sl* on Xpert dCE.

MTBDR*sl* on extract from cartridges done on clinical specimens. *TB detection.* As MTBDR*plus* was not feasible in the *in vitro* assessment, it was not done on CE from Ultras done on clinical sputa. MTBDR*sl*

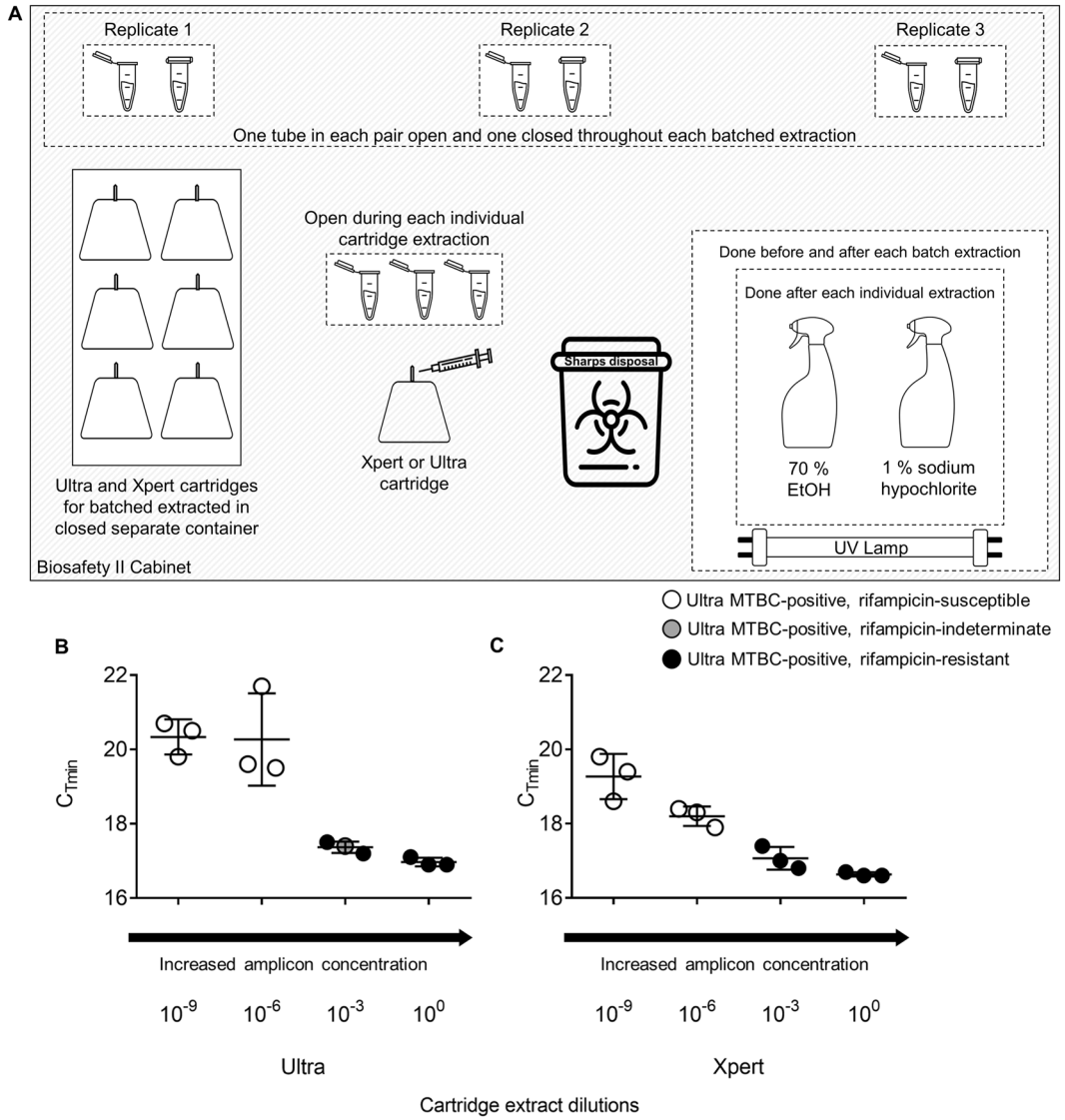

Figure 3. Evaluation of *rpoB* cross contamination risk experimental set-up and results. (**A**) Configuration of the environmental exposure experiment within a Biosafety level 2 cabinet. Three microcentrifuge tubes were open throughout each batched extraction procedure and three remained closed [median (IQR) extractions per batch 17.5 (10.5–27.5)]. No exposed tubes were FT *rpoB*-positive. In parallel to evaluate if, in an absolute worst case scenario, *rpoB* cross-contamination was probable, dCE from a (**B**) Ultra or (**C**) Xpert done on a drug-resistant strain was added to a drug-susceptible strain and the resultant mixture tested by Ultra. When samples of DS-TB contained CE at higher concentrations (undiluted, 10^{-3}), false-resistant (solid black circles) or indeterminate rifampicin resistance (grey circles) are seen. All samples containing CE dilutions beyond 10^{-6} showed true rifampicin susceptibility (white circles). Error bars represent C_{Tmin} values for each dilution. Some images were obtained from the Noun Project: microcentrifuge tube (without changes), Anthony Ledoux, https://thenounproject.com/term/eppendorf/1699532/; spray bottle (without changes), John Winowiecki, https://thenounproject.com/search/?q=spray%20bottle&i=2236898; sharps container, Juicy Fish (with changes), https://thenounproject.com/term/hospital-waste-bin/2450390/; needle (without changes), Creative Mania; https://thenounproject.com/search/?q=injection&creator=2251916&i=2409865.

on dCE from Ultra done on clinical sputa had 37/40 (93%) actionable results (the rest were non-actionable). Non-actionable results corresponded to "trace" or "very low" semi-quantitative categories.

Resistance detection. Of the actionable results, 35/37 (95%) fell within the defined threshold (C_{Tmin} <25) and of these all FQ results were concordant with MTBDR*sl* on sputum and all but one SLID result were concordant (false-susceptible). Though this percentage is slightly higher than the number of patients with C_{Tmin} <25 in our setting, which was determined to be 86% (based on an evaluation of Ultra done in sympotmatic patients in primary care[26]), which further show that this approach would benefit the majority of patients in our setting. Of the 2/37 (5%) results that were actionable but fell above the defined threshold, one was concordant with MTBDR*sl* on sputa and one was indeterminate for FQs and discordant for SLIDs (false-resistant).

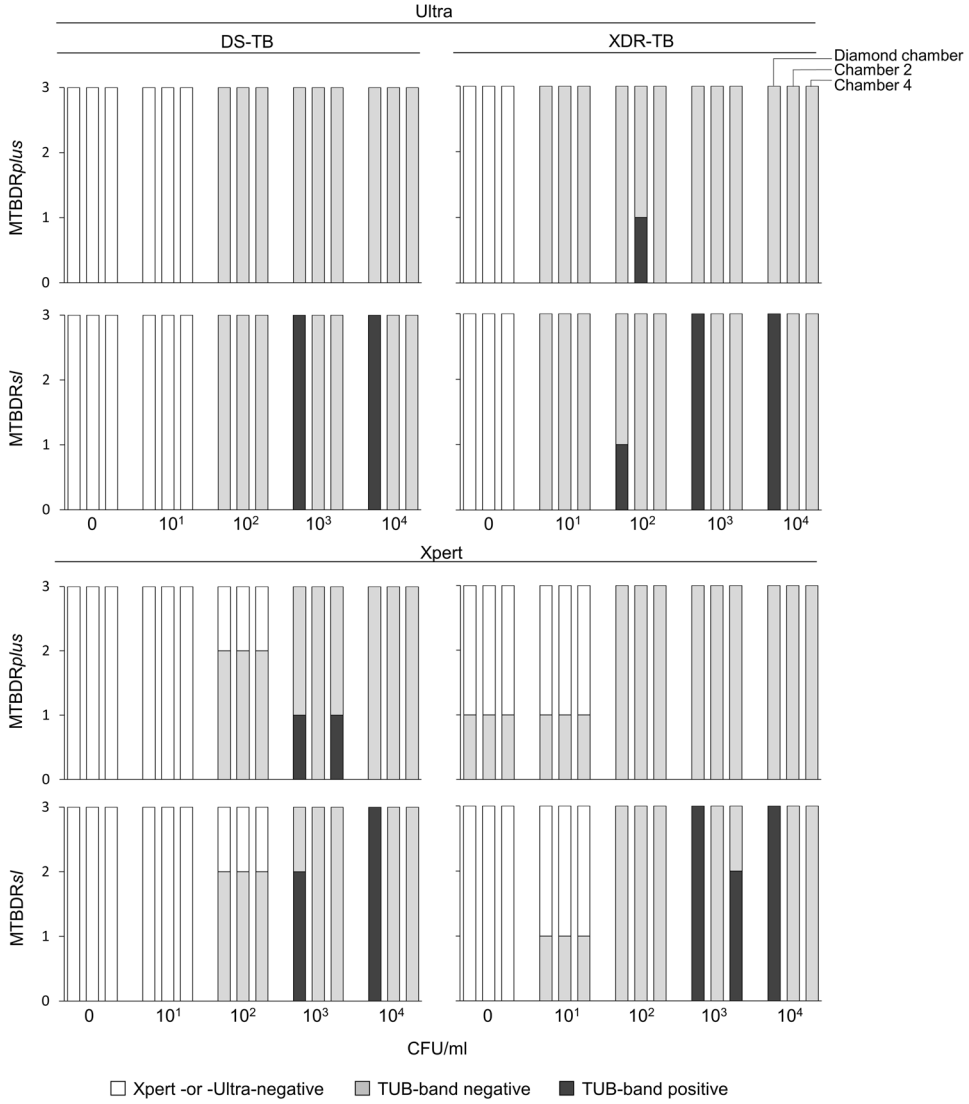

Figure 4. MTBDR*plus* and MTBDR*sl* on cartridge extract results for TB detection. dCE (left-most column), C2 (middle column) and C4 (right-most column) from *M. tuberculosis*-positive cartridges on dilution series (DS-TB and XDR-TB strains) are shown. MTBDR*plus* had mostly non-actionable results (not positive or negative). MTBDR*sl* had actionable results on all Ultra- and Xpert-positive dCE at $>10^3$. Though some actionable line probe assay results for non-diamond chambers were observed, these were inconsistent and had low reproducibility.

Receiver operator curve for determining actionable results. An Ultra *rpoB* C_{Tmin} threshold of <25.4 was defined for dCEs done on clinical sputa with sensitivities of 97% (95% CI 87–100) and specificities of 100% (55–100) (Fig. 6).

FluoroType MTBDR on extract from cartridges done on dilution series. *Diamond chamber.* FT had similar results to MTBDR*plus* on CEs. For example, 3/24 (12%) Ultra dCEs were MTBC-positive (the others negative) for both strains (Fig. S2). In the three Ultra dCEs with a TB-positive FT result, all had indeterminate susceptibility results for at least one drug. A total of 18/24 (75%) Xpert dCEs were FT MTBC-positive, however, of these 13/24 (54) were indeterminate for at least one drug.

Chamber 2. FT on Ultra C2 had MTBC positivity rates of 10/12 (83%) and 11/12 (92%) for DS-TB and XDR-TB, respectively. On Xpert C2, FT TB positivity rates were 5/12 (42%) and 7/12 (58%) for DS-TB and XDR-TB, respectively. In MTBC-positive extracts (Ultra and Xpert), most resistance calls were indeterminate or discordant with the paired isolate.

Chamber 4. FT done on C4 from Ultra had 8/12 (67%) and 9/12 (75%) TB positivity rates for DS-TB and XDR-TB strains respectively, and 3/12 (25%) and 1/12 (8%) on for C4 from Xpert respectively. As for C2, resistance calls were mainly indeterminate or discordant with paired isolate.

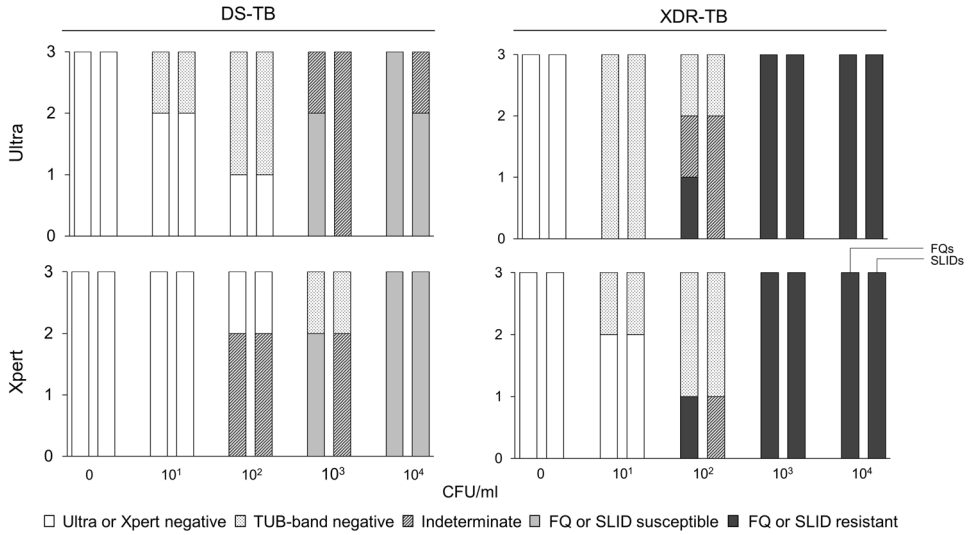

Figure 5. MTBDR*sl* drug susceptibility results on dCEs from Ultra and Xpert on dilution series. All results $\geq 10^3$ CFU/ml for the XDR-TB strain had resistance results concordant with the isolate. Some SLIDs indeterminate results were seen for the DS-TB $>10^3$ at the same concentrations but MTBDR*sl* results were otherwise concordant with those on the isolate.

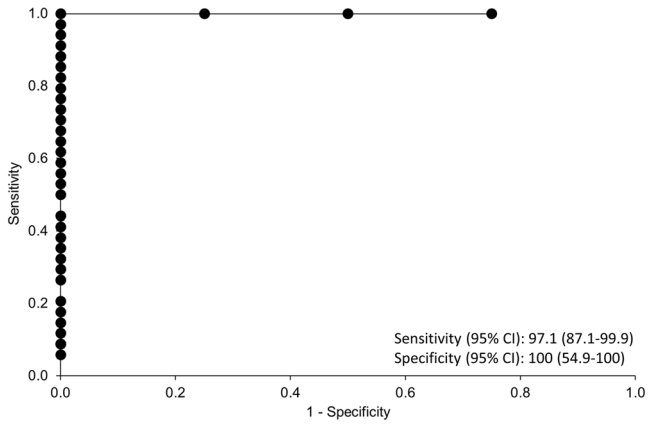

Figure 6. Receiver operation area under the curve of actionable vs. non-actionable results of MTBDR*sl* on Ultra diamond cartridge extract done on DR-TB clinical sputa to determine a C_{Tmin} threshold at which this approach is not feasible. MTBDR*sl* yields actionable results on cartridge extract from Ultra at a C_{Tmin} threshold of <25.4 with a sensitivity of 97% (87.1–99.9; 95% CI) and specificity of 100% (54.9–100; 95% CI) respectively.

***rpoB* amplicon cross-contamination risk evaluation.** *Exposure of open tubes during batched extractions.* All sixty tubes exposed were FT MTBC-negative and had no *rpoB* amplification.

Amplicon spiking for absolute worst-case cross-contamination scenario. Of the Ultra dCEs done on XDR-TB and spiked into DS-TB for re-testing with Ultra, evidence of cross-contamination was seen when dCEs were diluted less than 10^{-6} before addition to the DS-TB strain [3/3 (100%) of 10^0 dilutions and 2/3 (67%) of the 10^{-3} dilutions showed false-resistance (1/3 of the 10^{-3} was resistance indeterminate)] (Fig. 3B). Similar results were obtained for Xpert dCE (Fig. 3C).

Discussion

We have validated MTBDR*sl* on CEs from used Ultra cartridges for genotypic second-line DST. We show: (1) MTBDR*sl* on Ultra dCE when C_{Tmin} <25 enabled DST concordant with sputum results, (2) risk of *rpoB* extract cross-contamination is unlikely if standard aseptic protocols are followed, (3) neither 16S rRNA qPCR, MTBDR*plus*, MTBDR*sl* nor FT are feasible on other cartridge chambers, nor was MTBDR*plus* or FT on Ultra and Xpert dCEs. These data support the use of Ultra extract for second-line genotypic DST.

We defined a threshold at which MTBDR*sl* is likely to work on Ultra dCE from the vast majority of Ultra-positive patients, thereby avoiding time and resources wasted on dCE unlikely to give a valid result. We are mindful that there were some indeterminate SLID results (in line with previous reports of higher MTBDR*sl* indeterminate result rates for SLIDs vs. FQs)[27–29]. However, all dCE SLID-indeterminate results from the dilution series

were from the DS-TB strain and there were no indeterminate SLID results on XDR-TB dCEs. On clinical sputum (and falling within our threshold), one MTBDR*sl* SLID susceptibility result was discordant with sputum (one false-negative). We thus suggest that MTBDR*sl* Ultra dCE results are interpreted in the same manner as recommended by the WHO for MTBDR*sl* on clinical specimens[30]. If, for example, MTBDR*sl* on dCE is non-actionable or susceptible, MTBDR*sl* on sputum or isolates should be done. If there is still no evidence of resistance in a high burden setting, phenotypic DST should still be done given the suboptimal rule-out accuracy of MTBDR*sl*[19,30].

The possibility of contamination from *rpoB* amplicons during extractions has not been investigated. We implemented systematic testing for possible environmental contamination. No tubes exposed for each extraction batch were *rpoB*-positive when tested with FT. FT was used for testing for *rpoB* amplicons as it is more sensitive than MTBDR*plus*[14,15].

We further tested a worst-case contamination scenario with dCEs from both Ultra and Xpert cartridges done on a XDR-TB strain, diluting these dCEs, and adding them to a DS-TB strain which was subsequently tested by Ultra. The undiluted and most concentrated dCE dilutions (10^0, 10^{-3}) showed false rifampicin-resistance indicating that, although the GeneXpert platform does have proven ability to remove large numbers of amplicons[31], it was not able to remove all amplicons during the pre-amplification wash steps, however, amplicons diluted beyond 10^{-3} were successfully removed to the point of not being detected[22,32,33]. These results, together with those from the environmental samplings during extractions, shows that when standard aseptic techniques are used, amplicon cross-contamination is highly unlikely except in the artificial worst case scenarios. Finally, it should be noted that, in line with good practice in any molecular biology laboratory providing results for patient management, dCEs should not be collected in the same room where *rpoB*-based tests are done, and that the risk of cross-contamination from the dCE approach is only pertinent to tests for rifampicin resistance.

We suggest that diagnosticians considering implementing this approach use the cartridge itself as a transport vessel (upright and in sealed containers) to a central laboratory where dCE can be extracted appropriately (the diamond is a sealed chamber and should remain safe during transport). Most peripheral laboratories will be unable to do the dCE procedure safely and downstream molecular DST like MTBDR*sl*. This cartridge transport can interface with existing specimen referral networks. If dCE is planned purely for molecular epidemiology, we suggest that dCE be extracted and stored at −80 °C or alternatively the whole cartridge be stored at −20 °C until extractions can be done in a batched, centralised fashion. The long term stability of these approaches will require examination.

We further hypothesised that liquid from other cartridge chambers may avoid interference by *rpoB* amplicons. However, upon testing, this approach gave variable non-replicable results. This was true for qPCR, MTBDR*plus*, MTBDR*sl* and FT assays. This may also be due to very low concentrations of template in these chambers, for example C3 – which is the "wash chamber", and/or remnant PCR inhibitors (e.g., salts from the sample reagent). In light of this, we believe that the presence of these amplicons may prevent newer approaches, such as next generation sequencing methods, from performing well on dCE without to clean up steps. This warrants further investigation. CE from the diamond chamber hence remains the best option for downstream genotypic DST.

The results of this study should be interpreted within its limitations, namely aseptic techniques done in an assay- or procedure-specific biosafety cabinet are needed to minimise amplicon cross-contamination. However, this infrastructure should already be implemented per WHO guidelines[34] where LPAs are done routinely for patient care. Furthermore, per good laboratory practice, CEs should not be collected in the same room where *rpoB*- or *IS6110/1081*-based assays are done, nor should either procedure be done by the same personnel on a daily basis. Lastly, further investigation into cross-contamination risk should be done in a routine diagnostic setting. This should include multiple operators.

We also acknowledge that this method may increase risk of needle stick injury. Standard biosafety protocols should be strictly adhered to. We were recently funded to develop a device that can eject material from cartridges in a safe manner. Another limitation is MTBDR*plus* was not feasible on Ultra CEs and we suspect this is due to interference from both *rpoB* and IS*6110*/*1081* amplicons. Thus, combined with the large volumes (and hence diluted targeted DNA) recovered from non-diamond chambers in Ultra and Xpert, MTBDR*plus* (and also likely FT) on extract from any Ultra cartridge chamber is in all likelihood not useful for isoniazid or confirmatory rifampicin DST. Finally, although the diamond chamber is a closed system and appears protected against desiccation, we acknowledge that some desiccation may occur over prolonged periods that this requires future systematic evaluation. However, we recommend that extract method is done on an as fresh a cartridge as possible (either at a peripheral or central laboratory), in order to reduce the delays of DR-TB diagnosis. Formal evaluation of CE stability pre-extraction may be useful.

We conclude that dCEs from Ultra at the C_{Tmin} threshold (<25), can be used for genotypic second-line DST (MTBDR*sl*). Ultra and MTBDR*sl* on dCE therefore allows for the rapid rule-in detection of XDR-TB on a single specimen.

Data availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on request.

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Author contributions

G.T., R.W., M.D.V. and R.V. conceived the experiments. R.V., S.M., B.D., H.T., and A.R. conducted the experiments. T.D. provided specimens and data from the NHLS. R.V. and S.M. analysed data. All authors reviewed the manuscript.

Competing interests

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Chapter 4

High fidelity ultra-deep targeted sequencing of mycobacterial genomic DNA from used Xpert MTB/RIF Ultra cartridges enables single-specimen diagnosis of micro-heteroresistance

High fidelity ultra-deep targeted sequencing of mycobacterial genomic DNA from used Xpert MTB/RIF Ultra cartridges enables single-specimen diagnosis of micro-heteroresistance

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Abstract

Background: Full drug susceptibility testing (DST) is a key barrier to tuberculosis control. Mycobacterial genomic DNA from used Xpert MTB/RIF Ultra (Ultra) cartridges can be used for molecular tests like MTBDR*sl*, however, the feasibility of next-generation targeted sequencing methods like Single Molecule Overlapping Reads (SMOR), capable of detecting more targets and micro-heteroresistance (<1%), is unexplored. We evaluated if SMOR on cartridge extract (CE) from Xpert MTB/RIF (Xpert) and its successor Ultra was feasible.

Methods: Cartridges TB-positive and rifampicin-susceptible (n=27) or rifampicin-resistant (n=23) by Xpert and rifampicin-susceptible (n=32) or rifampicin-resistant (n=17) by Ultra had CE used for SMOR (*inhA*, *katG*, *rrs*, *eis*¸ *gyrA*). Where paired cultures were available had phenotypic DST (pDST). MTBDR*plus* and MTBDR*sl* results were collected from rifampicin-resistant patients.

Results: SMOR on Ultra CE had less actionable (i.e., presence of >10 total-reads and, if resistant, >0.1% resistance reads) reads vs. Xpert CE [13/49 (27%) vs. 27/50 (54%); p=0.005]. Ultra CE shotgun sequencing revealed multiple copies of *rpoB*, IS6160/1081, and *pncA* amplicons. C_{Tmin} thresholds at which SMOR yielded actionable results were <17 ("high") and <23 ("low") for Ultra and Xpert, respectively. SMOR calls were mostly concordant (compared to pDST or LPA) for Ultra and Xpert CEs for all drugs except rifampicin. However, SMOR detected non-*rpoB* heteroresistance missed by conventional DST in 9/99 patients [9%; 3/49 (6%) for Ultra, 6/50 (12%) for Xpert].

Conclusion: This proof-of-concept study shows SMOR on CE to be feasible; potentially leading to the full and rapid rule-in detection of second-line resistance on used Ultra cartridges, however, optimisation to reduce the potential interference from amplicons needs to be done.

Introduction

Tuberculosis (TB) is the leading infectious cause of death in the world and the emergence of drug-resistant (DR-) TB is a growing concern. Globally the incidence rate for reported DR-TB is 4% among new cases and 19% among previously treated cases but varies based on geographical location, for example it ranges from 3% and 7% in South Africa to 38% and 72% in Belarus, respectively [1].

Currently the World Health Organization (WHO) endorses the use of the Xpert MTB/RIF (Xpert) - now superseded by the Xpert MTB/RIF Ultra (Ultra) - and the line probe assays (such as MTBDR*plus* for detecting rifampicin and isoniazid resistance and MTBDR*sl* for detecting resistance to fluoroquinolones and second-line injectables) as molecular tests for drug susceptibility testing (DST). Conventional phenotypic testing on culture isolates (which is slow and requires biosafety infrastructure) is also recommended when results from molecular tests are inconclusive, discordant or, due to suboptimal sensitivity, susceptible[1]. However, despite recent improvements in detection of DR-TB due to molecular tests such as the LPAs, which reduce time-to-treatment-initiation, treatment initiation in programmatic conditions can still be as long as 55 days[2]. Only 22% of the estimated DR-TB cases have treatment success[1-5]. These delays are caused by many factors, including poor adherence to national diagnostic algorithms, suboptimal specificities and sensitives of tests, long incubation times for culture, and failure to acquire additional samples for patients for further testing[4,6-9].

Previously, a proof-of-concept study evaluated whether *Mycobacterium tuberculosis* genomic DNA was recoverable from the PCR-reaction mix in used Xpert cartridges (CE – cartridge extract) for downstream DST testing using MTBDR*plus*, MTBDR*sl* as well as spoligotyping[10]. It was found that CE from Xpert cartridges with a semi-quantitation result within the 'low' range and below a $C_{Tmin} \leq 24$, had MTBDR*sl* results concordant with that on sputa and that

spoligotyping was feasible. This potentially obviates loss in the patient diagnostic care cascade due to additional sputa not being available or initially submitted for DST.

Further exacerbating the problems facing DR-TB diagnosis are heteroresistant sub-populations of bacilli. These are often masked by larger populations of drug-susceptible bacilli and can remain undetected by both phenotypic and molecular DST techniques which leads to a delay in effective treatment. This in turn means the patient may remain infectious while a definitive diagnosis is sought and contribute towards transmission[11-15]. Furthermore, often patients are placed on ineffective treatment and allows for the drug-resistant sub-populations to proliferate, leading to poor patient outcomes[14,16]. The presence of resistant alleles is also often lost during sub-culturing which leads to inaccurate phenotypic DST results[13,17,18].

Previous studies done to determine the use of next-generation sequencing (NGS) using the single-molecule overlapping reads (SMOR) method are in theory able to result in fast and accurate drug resistance profiling to enable individualised treatment plans[19-21]. SMOR can also detect resistant alleles down to <1%[12,18]. While this method has been tested on various materials, it has never been assessed for use on CE.

We therefore tested the feasibility of SMOR on CE from used Xpert and Ultra cartridges to address several limitations that include: 1) the need to collect additional specimens for further sub-culture and DST if a patient has DR-TB 2) the inability of MTBDR*plus* to detect resistance to rifampicin and isoniazid on CE, and 3) detection of resistance-conferring mutations not in loci targeted by commercial molecular DSTs. We also explored if the addition of reagents to improve PCR amplification reduce interference from amplicons within used cartridges.

Material and Methods

Ethics statement

The study was approved by the Health Research Ethics Committee of Stellenbosch University (N09/11/296 and N14/10/136) and the City of Cape Town (#10570). Permission was granted to use anonymised residual specimens collected as part of routine diagnostic practice and thus informed consent was waived.

Definitions

An actionable result is defined as a result that can be used for clinical decision making (i.e. a susceptible or resistant result) where the total number of SMOR reads is >=10, and, if resistant, >0.1% proportion of the total reads are resistant. A non-actionable result is one that cannot be used for clinical decision making (i.e. it is indeterminate because it has <10 total reads and/or the resistant reads are <=0.1% of the total reads for that locus. C_{Tmin} – minimum cycle threshold value for Ultra or Xpert (proxy for bacillary load- a larger C_{Tmin} value corresponds to lower bacillary load and vice versa).

Sample collection

Used positive Xpert and Ultra cartridges done on sputa from people with symptoms suggestive of TB tested as part of the South African national TB diagnostic algorithm were collected consecutively between September 2016 and August 2017 from the National Health Laboratory Services (NHLS) and ongoing studies at Stellenbosch University, South Africa. Cartridges were stored at 4 °C for a maximum of 5 days prior to extraction as previously described[10]. Forty-nine Ultra cartridges were collected of which 32 were Ultra TB-positive rifampicin-susceptible and 17 were Ultra TB-positive rifampicin-resistant (Figure 1A). Fifty Xpert cartridges were collected in total, of which 27 were TB-positive, rifampicin-susceptible

cartridges and 23 were TB-positive rifampicin-resistant (Figure 1B). These included all semi-quantitation criteria for both Xpert and Ultra.

Phenotypic and genotypic drug susceptibility results

DST results from LPAs (MTBDR*plus* and MTBDR*sl*) done as part of the routine national algorithm for patients suspected of having DR-TB were collected from the NHLS for all rifampicin-resistant cartridges. Phenotypic drug susceptibility testing (pDST) was done on paired sputa that were collected where possible (Figure 1). For the rifampicin-susceptible cartridges, a subset collected were from the NHLS and unfortunately clinical isolates were not available for pDST. For those rifampicin-susceptible cartridges that were collected as part of ongoing studies, clinical isolates were available (as part of standard culture diagnosis) and were thus used for pDST for this study. Standardised pDST was done for rifampicin, isoniazid, amikacin, kanamycin, ofloxacin and pyrazinamide using a MGIT960 system with EpiCenter software as previously described (BD Diagnostics, USA)[22]. DSTs were performed using WHO-recommended critical concentrations (1.0 µg/ml for rifampicin,0.1 µg/ml for isoniazid, 2.0 µg/ml for ofloxacin, 1.0 µg/ml for amikacin, 2.5 µg/ml for kanamycin and 100.0 µg/ml for PZA)[23,24].

Single Molecule Overlapping Reads

A universal tail amplicon sequencing approach was used. Primers were designed to target six genes in *M.* tuberculosis[25-28]. These cover frequently-detected loci conferring resistance to isoniazid, rifampicin, ofloxacin, moxifloxacin, amikacin, kanamycin, and capreomycin. SMOR was used as previously described to achieve reads for each of the aforementioned resistance loci [21].

Whole genome shotgun sequencing on Ultra cartridge extract

Sequencing was performed as follows on one Ultra CE (rifampicin-susceptible, medium semi-quantitation) and one Xpert CE (rifampicin-susceptible, high semi-quantitation) to determine the possible cause of interference. ReSeqTB whole genome sequencing methods were done[29]. Briefly, approximately one microgram of DNA per sample was fragmented using a Q800R2 sonicator (QSonica, Newtown, CT, USA) with the following parameters: 3 minutes sonication with 15 seconds pulse on, 15 seconds pulse off, and 20% amplitude. The fragmented DNA was size selected to target 600-650bp by fragment separation using the Agencourt AMPure XP beads (Beckman Coulter). WGS DNA library preparations were performed using the NEBNextUltra II DNA Library Prep Kit for Illumina (New England BioLabs) with the following modifications. The adapters and 8 bp of index oligonucleotides (Integrated DNA Technologies, San Diego, CA) were used in place of those supplied in the NEB preparation kit [30]. A dual-indexing approach was utilized[31]. The samples were sequenced on an Illumina NextSeq using either a 300 cycle v2 mid output kit (Illumina) with the standard Illumina procedure. The appropriate sequencing primers were added to the cartridge[30].

ThermoGo and ThermoStop to improve sequencing reads on cartridge extract

ThermoGo is a modified nucleic acid that supresses mispriming and ThermoStop is a reversible hot start agent that prevents non-specific enzyme activity. These were both added to a subset of cartridge extract from both Xpert and Ultra as per manufacturer's instructions to assess whether this could increase the number of reads[32,33].

Results

actionableSMOR read counts and actionable results on Ultra and Xpert

SMOR reads per locus

When SMOR reads per loci were compared for each patient from Ultra-positive CEs, the median (IQR) reads were, from highest to lowest, *rpoB*: 19317 (210-27078), *gyrA*: 33 (4-2008), *katG*: 31 (5-3270), *inhA*: 9 (3-32), *rrs*: 3 (1-180), *eis*: 1 (1-96) (Table 1, which also includes SMOR read counts on Xpert CE). Significant differences were seen between loci (Figures 2A and 2B. Ultra had significantly less reads for *inhA*, *katG*, *gyrA*, *eis* and *rrs* than SMOR on Xpert CEs (Table 1).

SMOR reads as a function of bacillary load (C_{Tmin})

SMOR reads on Ultra CE did not correlate with bacillary load (Figure 3A), suggesting interference. SMOR on Xpert CE showed a significant positive correlation with C_{Tmin} increase [Spearman's rank correlation coefficient: r (CI 95%) = -0.6 (-0.7 – 0.3); p=0.001) with the exception of 4 samples that had non-actionable results at lower C_{Tmin} values (Figure 3B).

Actionable vs non-actionable results

Ultra had overall less actionable results compared to Xpert [13/49 (27%) vs 27/50 (54%); p=0.005]. Per loci, SMOR on Ultra CE had significantly less actionable results for *gyrA*, *eis* and *rrs* compared to Xpert CE (Figure 4A, Table 1). Non-actionable results were found across all semi-quantitations. All actionable results corresponded to "high" and "medium" semi-quantitations (and one "Trace" result). A C_{Tmin} threshold at which SMOR on Ultra CE would be actionable was defined to be < 17.45 (high semi-quantitation) with sensitivities and specificities of 77% and 97% respectively (Figure 3A).

Xpert CEs had SMOR actionable results for and non-actionable for 23/50 (46%) (Figure 4C). All SMOR non-actionable results corresponded to the "medium", "low" and "very low" Xpert

semi-quantitations (with the exception of one being a “high” semi-quantitation). Actionable results had mostly “high” and “medium” results and three “low” results. A C_{Tmin} threshold at which SMOR on Xpert CE would be actionable vs non-actionable was defined to be < 23.25 (“low” semi-quantitation) with sensitivities and specificities of 88.9% and 73.9% respectively (Figure 4D).

SMOR resistance detection on CE

Standard DST results

Of the 49 Ultra CEs, 32 were rifampicin-susceptible and 17 were rifampicin-resistant. Of the 32 rifampicin-susceptible samples, 8 (25%) had pDST data for rifampicin and isoniazid only and 16/32 (50%) had pDST data for rifampicin and isoniazid, fluoroquinolones (FQs) and second-line injectables (SLIDs). Of the rifampicin-resistant samples, 17/17 (100%) pDST and LPA data was collected (Figure 1A).

Of the 50 Xpert CE samples, 27 were rifampicin-susceptible and 23 were rifampicin-resistant. Of the 27 rifampicin-susceptible samples, only 10 (37%) had pDST data for rifampicin and isoniazid. Of the rifampicin-resistant samples, 19/23 (83%) and 23/23 (100%) had pDST and LPA results for rifampicin, isoniazid, FQs and SLIDs respectively (Figure 1B). Results below only include results for which there were DST data (pDST or LPA) available.

Rifampicin (Figure 5; Table 2)

Of the SMOR on Ultra CE rifampicin results for which there was DST data, 24/43 (56%) were concordant and 20/44 (45%) were discordant. Interestingly, one Ultra rifampicin-resistant discordant result was a false-positive rifampicin-resistant result (CX404), as both pDST and LPAs indicated rifampicin-susceptibility, while SMOR detected various single nucleotide polymorphisms (SNPs) <1%, as well as 74% of the H526N and 24% of the L511P SNPs. Follow-up data from routine diagnostics done at a later time point as part of the national

algorithm indicated this patient was found to have a heteroresistant infection using standard LPA methods (this patient was also isoniazid-susceptible by SMOR but resistant using standard DST methods).

Most discordant rifampicin results corresponded to the rifampicin-susceptible Ultra CEs and were detected as either low-heteroresistant (LHR, < 1%) or heteroresistant (HR, < 10%) by SMOR (Figure 5), giving sensitivities and specificities of 94% and 30% respectively (Table 2). If a stricter threshold for SMOR-resistance call is defined as > 1% proportion of total reads with resistance markers (i.e. HR or R calls only), the numbers would change to 34/43 (79%) concordant and 9/44 (21%) discordant (p=0.0001) with sensitivities and specificities of 94% and 66% respectively. Similarly for SMOR on Xpert CE, 27/48 (56%) were concordant with a sensitivity and specificity of 94% and 21% respectively and if the > 1% criteria were applied these would change to 94% and 66% respectively (p=0.0001).

Isoniazid

SMOR targeting *inhA*-promoter and *katG* had 17/49 (37%) actionable results with DST data for Ultra CE. Of these 15/17 (88%) were concordant with pDST and/or LPA results and 2/17 (12%) were discordant [one was false-resistant (high semi-quantitation)] and one false-susceptible [(medium semi-quantitation, this patient was later diagnosed with heteroresistant infection as mentioned above)] resulting in a specificity of 94%. Of the 13 isoniazid-resistant samples detected by pDST and/or LPA, none were actionable and so sensitivity could not be determined.

Of the Xpert TB-positive CEs with available DST data, 23/30 (77%) were actionable for isoniazid. Of these, SMOR was concordant with 21/23 (91%) of samples and discordant with 2/23 (9%) (both were false-positives with “low” and “very-low” semi-quantitation respectively) resulting in sensitivities and specificities of 100% and 78% respectively. Changing the threshold for SMOR-resistance calls to > 1% did not impact the results.

Fluoroquinolones

Of the SMOR on Ultra CE actionable results with pDST or LPA data collected 15/35 (43%) were actionable and all were found to be concordant. As none of these were resistant, sensitivities could not be calculated but specificity was 100% (15/15).

There were 18/23 (78%) results that were actionable for FQs on Xpert CE and had DST data available. Of these, SMOR was concordant with 16/18 (89%). SMOR detected false-resistance in for 2/18 (11%) of the CEs where the LPA was susceptible (one was low and one medium semi-quantitation). Of the two discordant samples one was LHR, and so if the threshold for SMOR-resistance call is applied these number change to 17/18 (94%) concordance. The one Xpert CE sample that was FQ resistant according to LPAs was SMOR non-actionable and so sensitivities could not be calculated, specificity was 89% (16/18) (unchanged when stricter criteria was applied).

Second-line injectables

SMOR targeting *rrs* and *eis*-promoter had 12/36 (33%) actionable results with DST data available of which 11/12 (92%) were concordant (one had LHR detected for capreomycin by SMOR but was susceptible for amikacin and kanamycin; high semi-quantitation). Again sensitivity could not be calculated but specificities were 92% (11/12) and 100% (12/12) respectively for < 1% and >1% SMOR-resistance call threshold categories respectively.

SMOR for SLIDS on Xpert CE had 20/23 (87%) actionable results with DST data available. Of these 18/20 (90%) were concordant and 2/20 (10%) were discordant [one false positive (low semi-quantitation) and one false negative (very low semi-quantitation)]. The false-positive result was classified as heteroresistant by SMOR with 1.89% of the population showing a mutation in G1484T *rrs* mutation. The false-susceptible result was detected for a patient who had XDR-TB infection (as classified by national algorithm). The semi-quantitation was very low (C_{Tmin} = 31.9) which is likely the reason for discordance.

Whole genome (shotgun) sequencing on Ultra cartridge extracts

Whole genome shotgun sequencing (WGS) revealed Xpert CE had large amounts of *rpoB* amplicons as expected. Ultra CE, in addition to *rpoB* amplicons, had the presence of *IS6110*/*1081* amplicons as well as *pncA* amplicons (Figure 4). Notably there were 5 different IS6110 amplicon sizes.

ThermoGo and ThermoStop on cartridge extract

The combination of ThermoGo and ThermoStop did not significantly improve the total number of actionable results. It did, however, improve the number of reads in some samples regardless of semi-quantitation or C_{Tmin} value. The total number of reads were only significantly improved for *rpoB* Xpert CE (100%, p= 0.002) and Ultra CE *rpoB* and *rrs* (94% and 76% respectively; p-value = 0.001 both) (Table 3).

Discussion

We have previously shown that genomic DST could be done on Xpert CE. We have now shown that targeted deep sequencing is 1) feasible on Xpert CE at a similar C_{Tmin} that was previously defined (at least medium semi-quantitation), 2) that it was also feasible on Ultra CE but had more non-actionable results and only actionable at low C_{Tmin} (high bacillary load), 3) WGS revealed that in addition to interfering *rpoB* amplicons, Ultra CE also contained various other amplicons that potentially confound SMOR, and 4) SMOR detected heteroresistance missed by conventional tests and had good specificities for resistance detection making it a good rule-in test.

As hetero-resistance is becoming a growing concern, new tools for detection of underlying sub-populations of resistant infections that may be missed by conventional molecular tests is needed. Targeted deep sequencing methods such as SMOR allows for detection of heteroresistance <1% which would be useful in a per-patient diagnostic approach, especially in cases where patients have had previous TB and may be failing treatment or have relapsed. Often it is difficult to collect more than one sputum for patient or to wait for culture to become positive therefore it would be very convenient to sequence straight from CE. We have shown that it is possible to do SMOR on Xpert CE at a similar C_{Tmin} that was defined previously for molecular tests, and can be used to rule-out samples on which SMOR in unlikely to work.

While SMOR did detect resistance missed by conventional tests, most fell above defined C_{Tmin} threshold. Further investigations should be done to determine the clinical relevance of the findings. While sensitivities could not be calculated for certain resistance conferring loci, non-*rpoB* specificities were good and indicates that SMOR could be used as a rule-in test and when susceptible results are obtained, the same procedure for LPAs as outlined by national algorithms is followed. Given the high proportion of false-resistant *rpoB* results, likely due to

amplicon interference from tests, it is suggested that rifampicin results from SMOR not be taken as actionable.

SMOR on Ultra CE worked less well, although high sensitivities and specificities were detected, the cut-off threshold category for which SMOR on Ultra would be actionable was 17.45 (high semi-quantitation) and is not representative of results in most clinical settings. WGS revealed that in addition to the *rpoB* amplicons, Ultra CE had *pncA*, *IS6110* and *IS1081* amplicons as well which maybe be causing additional interference in the sequencing. Curiously, the total read counts for *rpoB* were still higher than the multi-copy *IS610/1081* amplicons. This could be due to various reasons such as unforeseen dynamic with the spreading of *IS6110* primers across multiple sites, that there are multiple degenerate primer sites with minor variations in the *IS6110* target regions thereby decreasing amplification efficiency, that this sample behaved differently because it had a very high TB load or could also be that the *rpoB* primers are amplifying something in addition to *Mtb*. The addition of ThermoStop and ThermoGo did not improve reads in a predictive way other than for *rpoB* and this might once again only be due to large amounts of *rpoB*-amplicons in the CE.

The results of this preliminary study should be interpreted within the context of the limitations. We only had one Xpert CE sample with known FQ or SLID resistance to compare SMOR resistance detection to, for which SMOR was non-actionable. While we did have follow-up data for some patients, this was not the main aim of the study and further investigations to the clinical relevance detected by SMOR should be done. Further optimisation for SMOR CE should also be done, this will include reducing or removing the interfering amplicons[34].

This study shows promise for future testing for low level drug-resistance using one sample per patient which can be used to inform per patient treatment, especially for patients that have had previous TB are failing on their current treatment.

Figures

Figure 1: Flow-diagram of sample collection for **[A]** Ultra and **[B]** Xpert cartridges. Cartridges were either collected as part of programmatic testing for the national algorithm or as part of several ongoing studies at Stellenbosch University (SU), South Africa. Cartridge extract was recovered from all collected cartridges and was used for Single Molecule Overlapping Read (SMOR) analysis. As per the national algorithm, all samples that are Ultra or Xpert rifampicin-resistant get follow up DST with LPA testing for first and second-line drugs (MTBDRplus, MTBDRsl; grey dashed-arrows). All resistant isolates collected and sent to SU were used for pDST as part of ongoing studies (rifampicin, isoniazid, ofloxacin, amikacin and pyrazinamide). Ultra and Xpert rifampicin-susceptible cartridges were collected where possible as part of other ongoing studies and used for subsequent pDST (either for rifampicin and isoniazid, or for rifampicin, isoniazid, ofloxacin, kanamycin, amikacin and pyrazinamide) where possible. SMOR, LPA results and pDST results (grey boxes, grey arrows) were compared. Abbreviations: NHLS – National Health Laboratory Service, CE – cartridge extract, R – resistant, S – susceptible, RIF – rifampicin, INH – isoniazid, OFX – ofloxacin, AMK – amikacin, KAN – kanamycin, PZN – pyrazinamide, pDST – phenotypic drug susceptibility testing, LPA – line probe assay (MTBDR*plus*, MTBDR*sl*).

Figure 2: Total reads across all patients and all loci (*rpoB*, *inhA*, *katG*, *gyrA*, *eis*, *rrs*) detected by SMOR vs C_{Tmin} per loci for **[A]** Ultra and **[B]** Xpert cartridge extract. Total reads shows trend of declining with increase in C_{Tmin} (i.e. decrease in bacillary load), this occurs more rapidly for SMOR on Ultra than for Xpert (with the exception of 4 samples). p-values indicate Spearman's correlation coefficient. Dotted lines represent 95% CI.

Figure 3: Total reads detected by SMOR per loci (*rpoB*, *inhA*, *katG*, *gyrA*, *eis*, *rrs*) for **[A]** Ultra and **[B]** Xpert cartridge extracts. Similar results were seen for all loci (except *rpoB*) but

significant differences were seen between loci. **** p-value <0.0001, *** p-value <0.001, ** p=< 0.01, * p=<0.05.

Figure 4: [A] SMOR on Ultra CE had actionable results for 13/49 (27%) of results and 36/49 (73%) non-actionable results (p=0.001) All of the actionable results had "high" or "medium" semi-quantitation categories (and one "Trace"). Non-actionable span across all semi-quantitation categories. **[B]** *rpoB* C_{Tmin} threshold of < 17.45 (Youden's index) was determined for when SMOR on Ultra would yield actionable results with a sensitivity and specificity of 76.9% [IQR (%): 50.5-93.4] and 97.2% [IQR (%): 87.5 - 99.9] respectively. This corresponds to the "high" semi-quantitation result. **[C]** SMOR on Xpert CE had actionable results for 27/50 (55%) of results and 23/50 (45%) non-actionable results (p=0.42) with a majority of the actionable results corresponding to "medium" and "high" semi-quantitation categories. **[D]** A C_{Tmin} threshold of < 23.25 SMOR on Xpert was determined to yield actionable results with a sensitivity and specificity of 88.9% [IQR (%): 73.7-96.9] and 73.9% [IQR (%): 54.9 – 88] respectively. This corresponds at least a "medium" and some "low" semi-quantitation results. Overall SMOR on Ultra CE had significantly less actionable results than Xpert CE (p=0.005). Dotted lines represent 95% CI. Abbreviations: SMOR – single molecule overlapping reads, CE – cartridge extract, C_{Tmin} – minimum cycle threshold value , AUC – area under the curve, PPV – positive predictive value, NPV – negative predictive value.

Figure 5: SMOR on Ultra CE had more non-actionable results for Ultra (p=0.005). There were several non-*rpoB* discordant results [9/99 (10%); 3/49 (6%) and 6/50 (12%) for Ultra and Xpert respectively; black circles] where SMOR detected resistance but conventional DST missed and one instance [1/99 (1%)] where SMOR was susceptible but conventional DST were resistant (very low semi-quantitation). Corresponding semi-quantitations can be seen next to discordant results. SMOR results per patient shown as either low heteroresistant (LHR; light pink squares), heteroresistant (HR; dark pink squares), resistant (R; red squares), susceptible, (S, green

squares) or non-actionable (grey squares). Concordance (hollow circle) or discordance (solid black circle) with either pDST and/or LPA results as reference (or only Xpert-rifampicin results as reference for XX700-XX726) for Ultra and Xpert CE are shown across both rifampicin-resistant –and -susceptible results. Asterix marks cases with special circumstances – CX404 was Ultra and SMOR rifampicin-resistant but LPA -and pDST-susceptible as well as SMOR isoniazid-susceptible but LPA -and pDST-resistant, results done on specimens collected later as part of national algorithm indicated the patient had a heteroresistant infection. XX927 was Xpert rifampicin-susceptible but resistant on LPA and pDST, SMOR detected the presence of a 24% HR population for this patient. There were some patients where LPA and pDST data were unavailable or inconclusive (black cross). Abbreviations: RIF – rifampicin, INH – isoniazid, FQ – fluoroquinolones, SLID – second-line injectables, pDST – phenotypic drug susceptibility testing, LPA – line probe assay (MTBDR*plus*, MTBDR*sl*), CE – cartridge extract, H – high, M – medium, VL – very low semi-quantitation.

Figure 6: Results of whole genome sequencing on **[A]** Ultra cartridge extract which revealed mainly *rpoB* amplicons as well as *pncA, IS6110* and *IS1081* amplicons. Notably there were 5 different sizes of *IS6110* amplicons **[B]** Xpert cartridge extract was revealed to only contain *rpoB* amplicons.

Figure 1

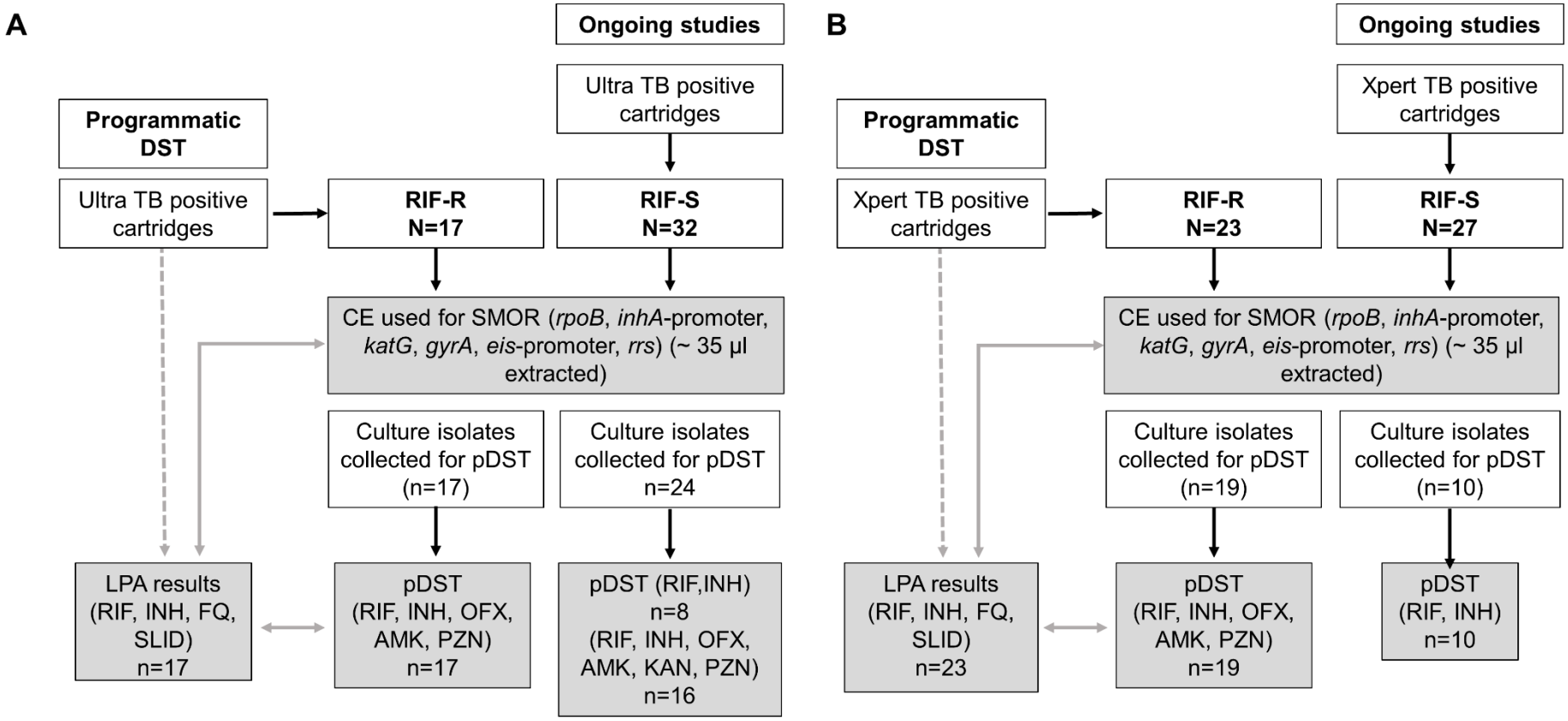


Figure 2

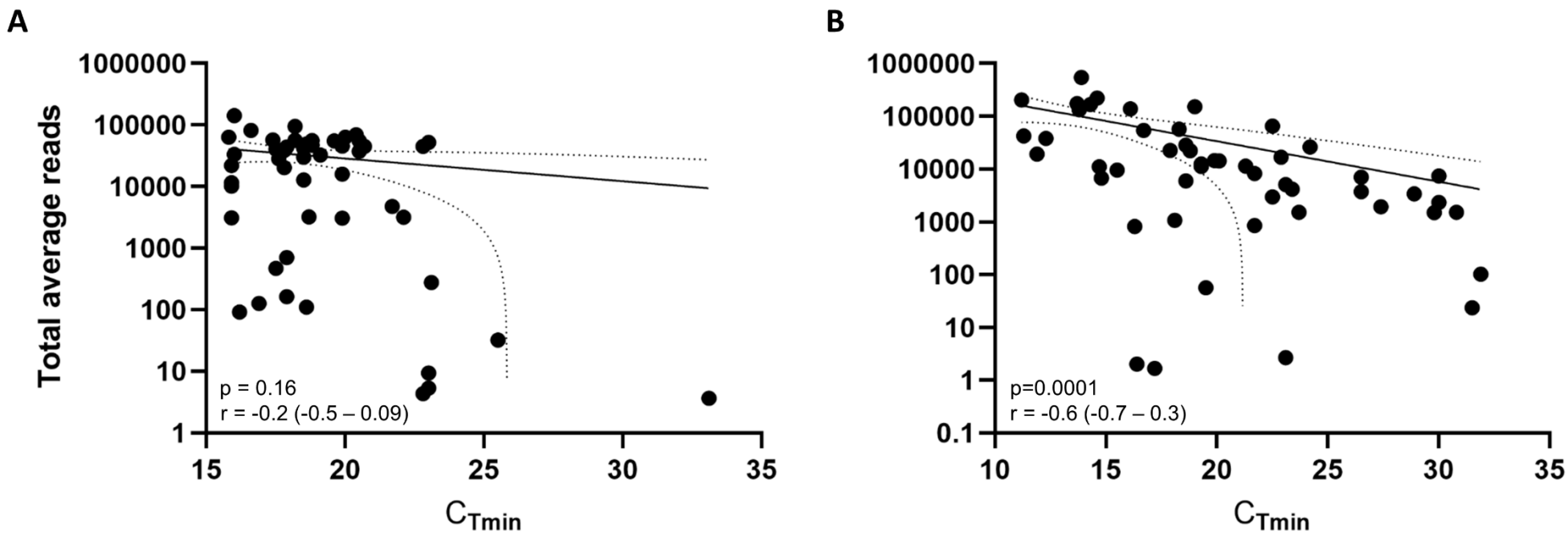


Figure 3

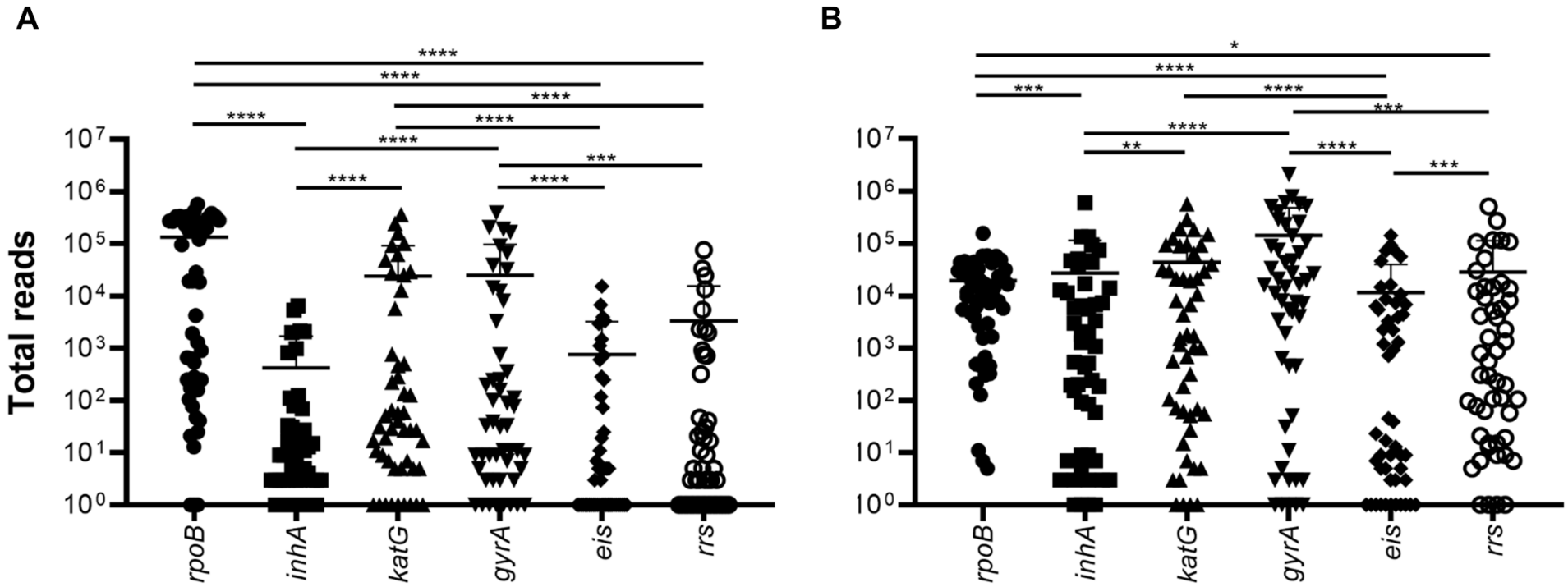
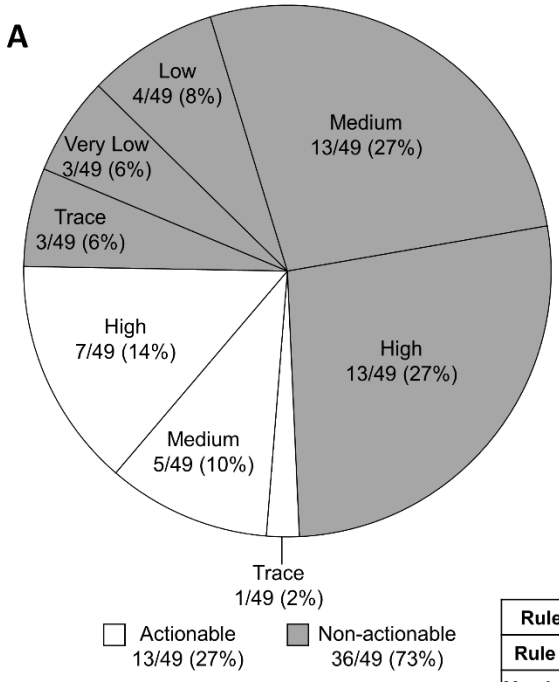
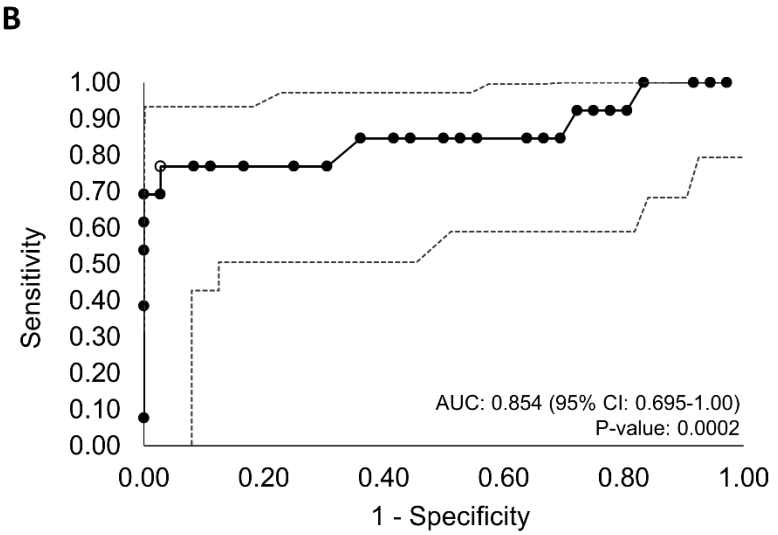
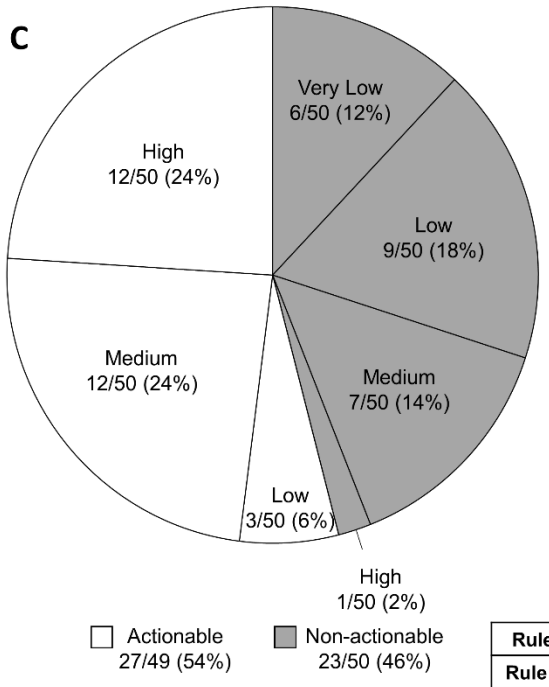


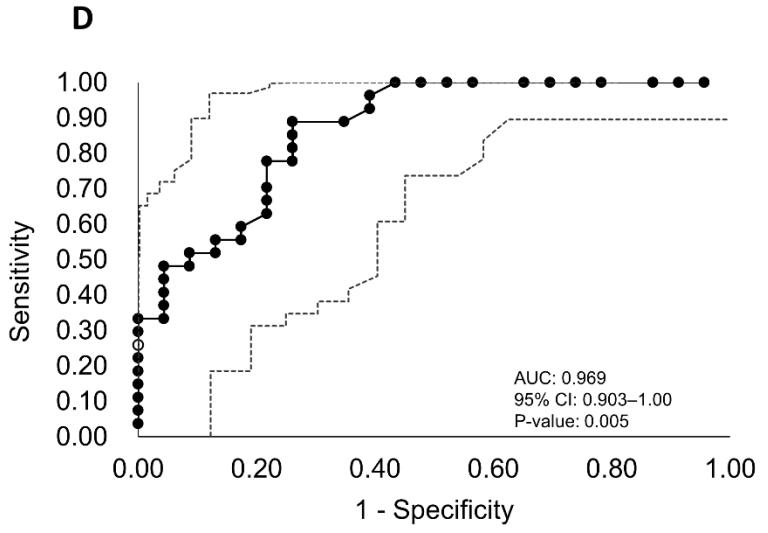
Figure 4





	C_{Tmin}	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)
Rule in test	<16.75	69.2 (42.7 - 88.7)	100 (92 - 100)	100 (71.7 - 100)	90 (78.6 - 96.5)
Rule out test	<22.9	100 (79.4 - 100)	16.7 (7.5 - 30.3)	30.2 (18.9 - 43.7)	100 (60.7 - 100)
Youdens index	<17.45	76.9 (50.5 - 93.4)	97.2 (87.5 - 99.9)	90.9 (63.6 - 99.5)	92.1 (80.8 - 97.8)





	C_{Tmin}	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)
Rule in test	<14.65	33.3 (18.6 - 50.9)	100 (87.8 - 100)	100 (71.7 - 100)	56.1 (42.1 - 69.4)
Rule out test	<21.5	100 (89.5 - 100)	56.5 (37.5 - 74.2)	73 (58.5 - 84.5)	100 (79.4 - 100)
Youden's index	<23.25	88.9 (73.7 - 96.9)	73.9 (54.9 - 88)	80 (64.3 - 90.9)	85 (65.6 - 95.8)

Figure 5

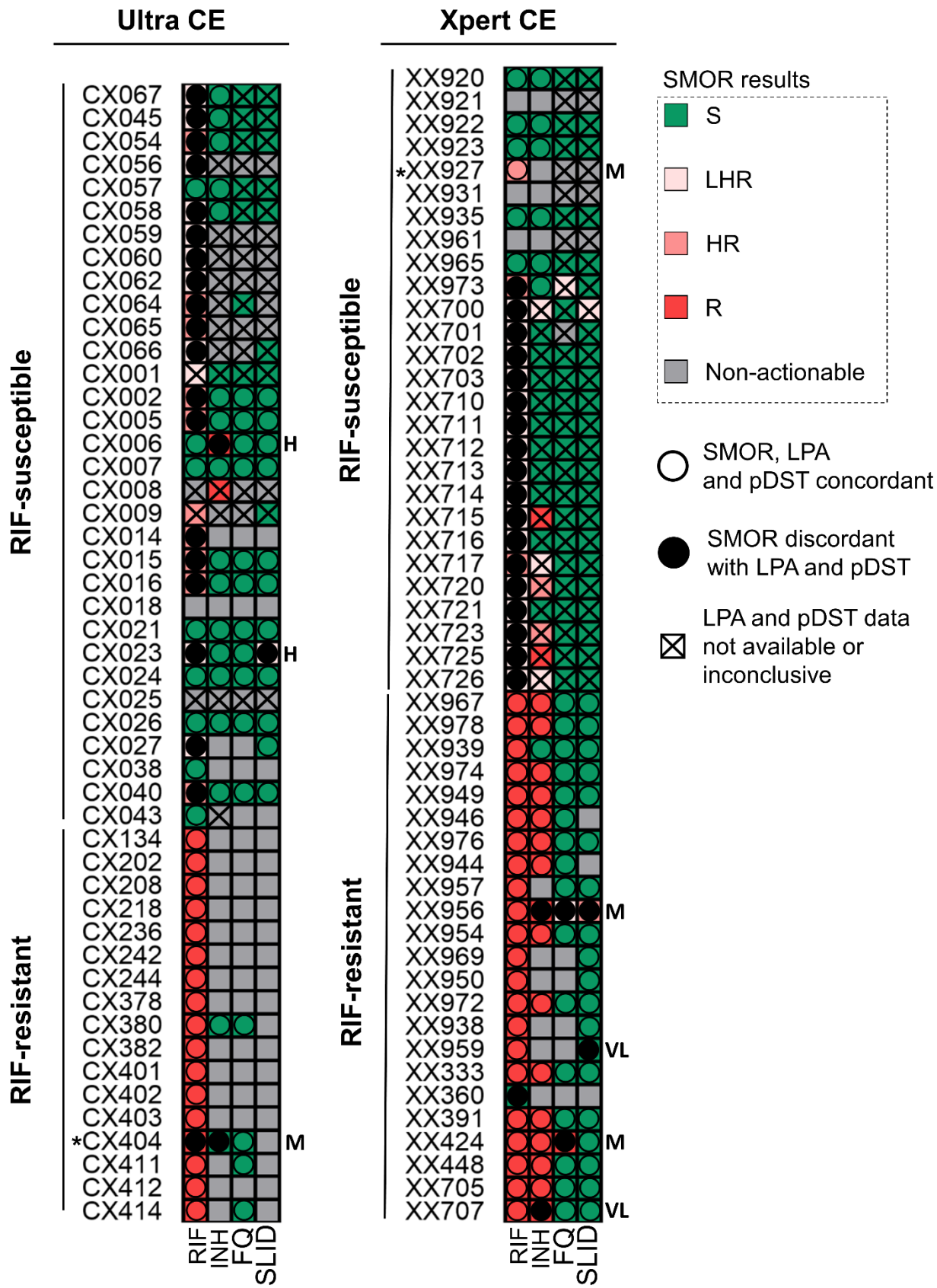
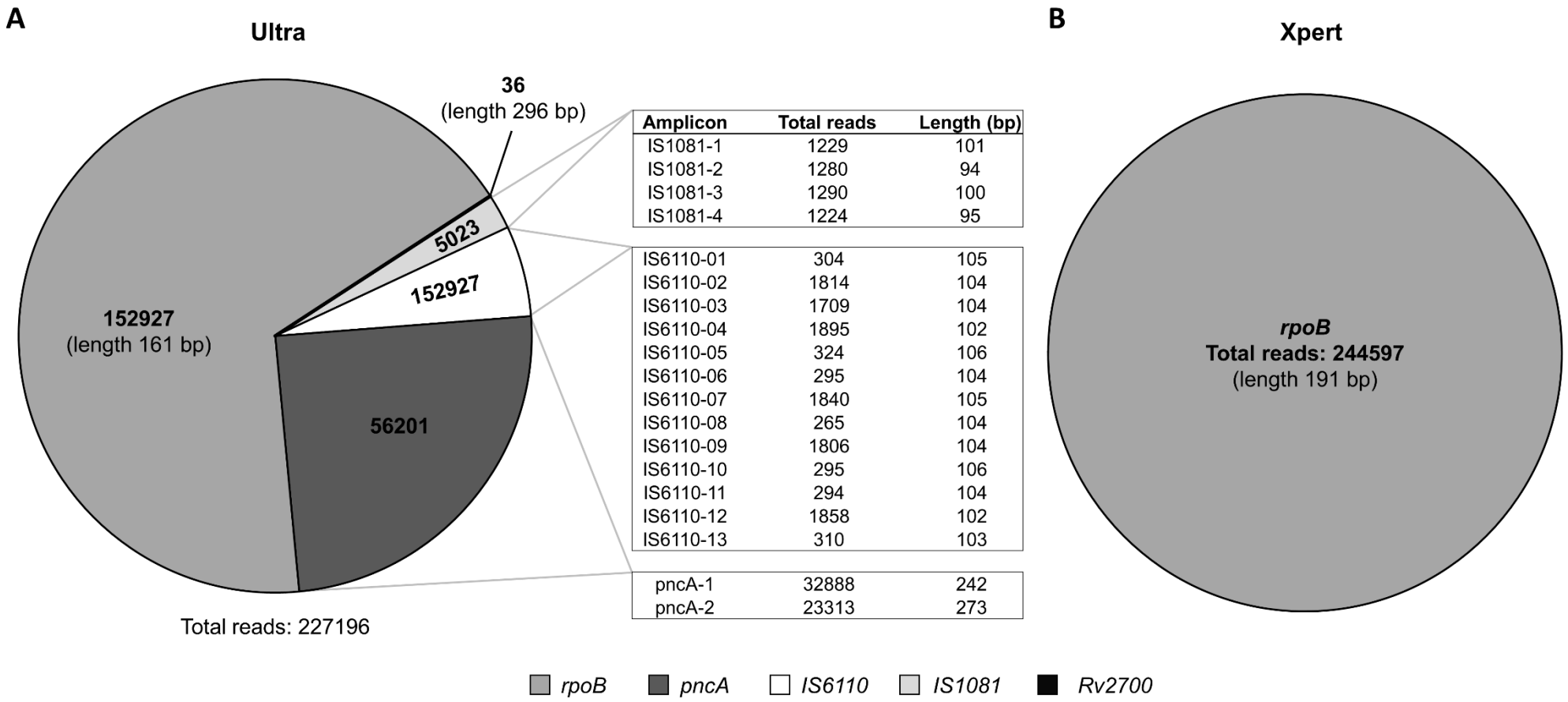


Figure 6



Amplicon	Total reads	Length (bp)
IS1081-1	1229	101
IS1081-2	1280	94
IS1081-3	1290	100
IS1081-4	1224	95
IS6110-01	304	105
IS6110-02	1814	104
IS6110-03	1709	104
IS6110-04	1895	102
IS6110-05	324	106
IS6110-06	295	104
IS6110-07	1840	105
IS6110-08	265	104
IS6110-09	1806	104
IS6110-10	295	106
IS6110-11	294	104
IS6110-12	1858	102
IS6110-13	310	103
pncA-1	32888	242
pncA-2	23313	273

Tables

Table 1: Total SMOR reads per loci on CE and the proportion of results for which SMOR gave an actionable result (≥ 10 reads, and > 0.1% resistance detected) for Ultra and Xpert (Ultra and Xpert not done on same patient sputa). Xpert had higher reads than Ultra for all loci except *rpoB* and Xpert had overall a higher proportion of actionable SMOR results than Ultra (this was driven by additional actionable results predominantly in the *gyrA*, *eis* and *rrs* loci). P-values represent Mann Whitney test (for total reads per loci between Ultra and Xpert) and test of proportions (for proportion actionable reads between Ultra and Xpert).

	Total reads (median, IQR)					
	rpoB	***inhA***	***katG***	***gyrA***	***eis***	***rrs***
Ultra	19317 (210-27078)	9 (3-32)	31 (5-3270)	33 (4-2008)	1 (1-96)	3 (1-180)
Xpert	10615 (1630-30350)	803 (9-13224)	1601 (54-33845)	12924 (46-94098)	31 (3-6415)	431 (18-12563)
p-value	0.20	**0.0001**	**0.005**	**0.0005**	**0.0004**	**0.0001**
	Total proportion actionable reads					
Ultra	46/49 (93%)	22/49 (45%)	33/49 (67%)	29/49 (59%)	16/49 (32%)	20/49 (40%)
Xpert	48/50 (96%)	36/50 (72%)	41/50 (82%)	40/50 (80%)	28/50 (56%)	41/50 (82%)
p-value	0.62	0.09	0.42	**0.02**	**0.02**	**0.0001**

Table 2: Results for which SMOR on CE was concordant and discordant for samples for which DST (either phenotypic or LPA) results were available and sensitivity and specificity of SMOR based on DSTs as reference standard. Data is shown [% (N/N)] for when "true" resistance is classified as including < 1% resistance and >1 % resistance detected. Certain sensitivities could not be calculated as true resistant samples were either non-actionable or, in one case, detected as susceptible by SMOR. SMOR was mainly concordant on all non-*rpoB* loci resulting in strong specificities for both Ultra and Xpert, making it a candidate for a rule-in test.

	Resistance < 1%				**Resistance > 1%**			
	RIF	**INH**	**FQ**	**SLID**	**RIF**	**INH**	**FQ**	**SLID**
Ultra CE								
Discordant	44 (19/43)	12 (2/17)	0 (0/15)	8 (1/12)	21 (9/43)	12 (2/17)	0 (0/15)	**0/12 (0)**
Concordant	56 (24/43)	88 (15/17)	100 (15/15)	92 (11/12)	79 (34/43)	88 (15/17)	100 (15/15)	**12/12 (100)**
	p=0.28	**p=0.0001**	-	**p=0.0001**	p=0.0001	**p=0.0001**	-	
Sensitivity	94 (16/17)	0 (0/1)	-	-	**94 (16/17)**	0 (0/1)	-	-
Specificity	30 (8/27)	94 (15/16)	100 (15/15)	11/12 (92)	**66 (18/27)**	94 (15/16)	100 (15/15)	**100 (12/12)**
Xpert CE								
Discordant	44 (21/48)	9 (2/23)	11 (2/18)	10 (2/20)	**43/48 (90)**	9 (2/23)	**6 (1/18)**	10 (2/20)
Concordant	56 (27/48)	91 (21/23)	89 (16/18)	90 (18/20)	**5/48 (10)**	91 (21/23)	**94 (17/18)**	90 (18/20)
	p=0.22	**p=0.0001**	**p=0.0001**	**p=0.0001**	**p=0.0001**	**p=0.0001**	**p=0.0001**	**p=0.0001**
Sensitivity	96 (23/24)	100 (4/14)	-	0 (0/1)	96 (23/24)	100 (4/14)	-	0 (0/1)
Specificity	21 (5/23)	78 (7/9)	89 (16/18)	95 (18/19)	91 (21/23)	78 (7/9)	89 (17/19)	95 (18/19)

Table 3: Effects of ThermoStop (TS) and ThermoGo (TG) on total reads (actionable and non-actionable) of SMOR on Ultra and Xpert CEs. The total amount of actionable reads did not improve with addition of TS and TG. However, reads improved overall for some samples. Significant differences were only seen for *rpoB* in both Ultra and Xpert CE as well as *rrs* in Ultra CE. P-values represent test of proportions (SMOR actionable rate without or with TS and TG) and Wilcoxon signed rank test. (SMOR total reads with or without TS and TG).

	Ultra						**Xpert**					
	rpoB	***inhA***	***katG***	***gyrA***	***eis***	***rrs***	***rpoB***	***inhA***	***katG***	***gyrA***	***eis***	***rrs***
SMOR actionable	14/17 (82%)	8/17 (47%)	14/17 (82%)	10/17 (59%)	9/17 (53%)	8/17 (47%)	10/10 (100%)	4/10 (40%)	9/10 (90%)	8/10 (40%)	2/10 (40%)	6/10 (40%)
SMOR actionable +TS and TG	14/17 (82%) -	9/17 (53%) p=0.73	15/17 (88%) p=0.62	13/17 (77%) p=0.27	9/17 (53%) -	8/17 (47%) -	10/10 (100%) -	5/10 (50%) p=0.65	9/10 (90%) -	7/10 (50%) p=0.61	3/10 (50%) p=0.61	4/10 (50%) p=0.37
Samples with total reads improved	16/17 (94%)	10/17 (59%)	8/17 (47%)	7/17 (41%)	7/17 (41%)	13/17 (76%)	10/10 (100%)	4/10 (40%)	4/10 (40%)	5/10 (50%)	4/10 (40%)	4/10 (40%)
Difference (reads; Median IQR)	21354 (1130-85719) **p=0.001**	10 (4-215) p=0.06	40 (7-12701) p=0.39	92 (9-25005) p=0.5	34 (3-228) p=0.6	6 (2-4608) **p=0.001**	158636 (144295-189789) **p=0.002**	13 (1.5-3298) p=0.38	47 (25-6380) p=0.63	5526 (20.5-43702) p=0.64	9 (1.5-261) p=0.87	16 (3.5-8367) p=0.91

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Chapter 5

Improving the utility of tuberculosis diagnostic tests for treatment monitoring

Improving the utility of tuberculosis diagnostic tests for treatment monitoring

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Abstract

Background: Methods to monitor drug-resistant TB treatment response, such as culture are slow and do not detect differentially culturable TB bacilli (DCTB) which are present in a dormancy-associated state. Furthermore, molecular methods are confounded by presence of DNA from dead bacilli which can lead to false positives. New methods, which include the use of exponential cell free extract (EPCFE) allow for detection of DCTB, or that render mycobacterial DNA from non-live cells non-amplifiable (which can improve the specificity of PCR tests), require evaluation.

Methods: Patients in a drug trial receiving the then-stand-of-care multi-drug resistant (MDR-) TB regimen or a shorter regimen were longitudinally-sampled. Sputum smear microscopy, MGIT960, the most probable number (MPN) assay (containing EPCFE or 7H9 broth and quantifies bacterial load) were each done (367 time points, 43 patients). Standard Xpert MTB/RIF (Xpert) and a modified viable PCR (vPCR) Xpert with a photoactive dye to reduce the amplification of DNA from non-intact cells were done (182 time points, 20 patients.

Results: MPN assay with added EPCFE detected more bacilli overall in both arms (but not between arms). Furthermore, MPN assay with EPCFE detected 14/47 (30%) and 9/41 (22%) positive patients in the routine and intervention arms respectively (missed by 7H9-negative). A subset of these patients were also missed by MGIT960 sputum culture [10/14 (71%) and 5/9 (55%) for each arm] and Xpert [2/8 (25%) and 2/6 (33%) for each arm; some Xpert results non-actionable]. MGIT960s was negative beyond week 24 but MPN had positive results at later time points, both with and without EPCE supplementation. Compared to culture and smear, Xpert had a high positive result proportions that were not reduced by the dye-based method.

Conclusion: EPCFE supplementation enables DCTB detection missed by other methods. The clinical importance of these populations requires clarification. The dye-based approach does not render Xpert useful for treatment monitoring.

Introduction

Tuberculosis (TB) is the biggest infectious cause of death worldwide[1]. Globally, the total incidence of drug-resistant TB is 558 000 and the treatment success rate for multi-drug resistant (MDR-) TB is 55% [34% for extensively-drug resistant (XDR-) TB], yet in some countries it is as low as 32%[2,3]. Poor treatment outcomes have been reported globally and are due to many factors, including HIV-coinfection as well as lengthy and ineffective treatment regimens[4-10]. It is therefore imperative to have accurate tools for monitoring treatment response to ensure patients remain on effective treatment, and reduce the chance of treatment failure and transmission from patients on treatment. New regimens for MDR-TB are desperately needed.

The current standard for treatment monitoring include sputum culture and smear, which are either slow or have relatively poor sensitivity and specificity for predicting treatment failure[11,12]. Culture can take up to 42 days for a result to be generated and is further confounded by the fact that there exists a sub-population of bacteria not culturable by conventional methods[13-15].

The proportion of differentially culturable tubercle bacilli (DCTB) has been found to increase through treatment. These bacilli are drug tolerant and detecting them is a challenge that requires modified culture methods[13,16]. Such methods may utilize exponential phase cell free extract (EPCFE) [which contains resuscitation promoting factors (RPFs) and other rescue proteins] to encourage DCTB growth[13,16]. These methods often have, as a readout, the Most Probable Number (MPN). The MPN assay is a common microbiological technique that is used to estimate the concentration of viable bacilli in a sample. This is done via a dilution series (which is done in replicate) and growth in each dilution is scored and used to back-calculate the total bacteria in the starter sample[17]. The MPN method can therefore be used to measure the culturability of different media against one another, for example to see if more bacilli are quantifiable in EPCFE supplemented broth versus standard 7H9 broth.

Xpert MTB/RIF (Xpert; Cepheid) is a nucleic acid amplification test (NAAT) and is endorsed by the World Health Organization (WHO). However, it is not currently recommended for this purpose as false-positive results may be obtained from DNA from non-viable bacilli which accumulate due to the bactericidal effects of the TB drugs[18-21]. This was demonstrated by Friedrich *et al*, where for example, at week 8 on treatment, culture had between 26%-42% positive results where Xpert had 84% positive results[18]. Novel, photoactive dyes such as propidium monoazide (PMA) and ethidium monoazide (EMA) have been used previously in a technique called viable PCR (vPCR) and was shown to intercalate with free DNA or DNA in non-intact cells through covalent bonds. This blocks DNA from being amplified[22-27]. When used in conjunction with Xpert, such techniques may improve diagnostic accuracy for treatment monitoring. Specifically, a novel double dye, PEMAX (produced by GenIUL, Spain) to overcome limitations of PMA and EMA such the inability to bind DNA from cells that are still intact but no longer metabolically active, may also improve specificity.

We therefore aimed to do a preliminary investigation to improve these two conventional diagnostics (culture, Xpert) and enhance their potential use for treatment monitoring.

Methods

Study design and ethics

Methods and protocols were carried out in accordance with relevant guidelines and regulations. The study was approved by the Health Research Ethics Committee of Stellenbosch University (M15/10/041).

Parent study

This study was nested within a funded clinical drug trial (NExT; Clinicaltrials.gov #NCT02454205) which evaluated a new multi-drug resistant MDR-TB regimen containing bedaquiline and linezolid (intervention arm; 6 month regimen) compared to the standard MDR-TB treatment (standard treatment arm containing fluoroquinolones and second-line injectables; 24 month regimen). All persons who were eligible for the Cape Town arm of the NExT study were also asked to participate in this study. Eligible persons were recruited from the Western Cape, South Africa from November 2015 to December 2018 (Table S1). NExT patients were followed up through treatment and post-treatment (Figure 1A). Three sputa were collected over the course of 48 hours and randomly used for culture and smear, MPN assay (both with EPCFE and standard 7H9) and Xpert (both vPCR-Xpert and standard Xpert) (Figure 1B).

Sputum processing

Culture and smear

Sputa were decontaminated using the N-acetyl-L-cysteine-sodium hydroxide (NALC-NaOH) method using the commercial kit MycoPrep (BD Biosciences, United States). Briefly, an equal volume of NALC-NaOH (0.5 M NaOH, 0.37 g/L N-acetyl-L-cysteine and 0.05 M trisodium citrate dehydrate) was added to the sample and vortexed, after a 15 min incubation at room temperature (RT), specimens were neutralized with phosphate buffer (PB) (33 mM Na_2HPO_4,

33 mM KH_2PO_4; pH 6.8) and centrifuged at maximum speed (4000 x g) for 15 min at RT. The pellets were resuspended in 1.5 ml PB, 500 µl was used for liquid culture inoculation using the MGIT 960 system (BD Biosciences) supplemented with 800 µl PANTA (containing an antibiotic mixture of polymyxin B, amphotericin B, nalidixic acid, trimethoprim and azlocillin; BD Biosciences). After 30µl was used for smear microscopy using Ziehl-Neelsen staining technique (ZN; acid fast stain), the rest of the specimen was biobanked at -20°C.

Most probable number assay

Sputa were decontaminated as described above. EPCFE was made as described [13]. Briefly, frozen stocks of 1 ml exponential phase culture (H37Rv, OD_{600} 0.6-0.8) were inoculated into 7H9 Middlebrook broth (BD Biosciences) and cultured to mid-exponential phase (OD_{600} 0.6-0.8). The liquid culture was centrifuged for 20 min at maximum speed (4000 x g) at RT and the supernatant was sterilized by passing it through 0.22 µm PES syringe filters twice. The EPCFE was supplemented in 1:1 ratio with fresh 7H9 liquid broth (7H9) and PANTA. The EPCFE mixture and fresh 7H9 with PANTA only was aliquoted into 48 well plates (Nunc, Thermo Fischer) (450 µl, three replicates rows each). A serial dilution was created across the plate using 50 µl of decontaminated sputa (Figure 2). Colony forming units were also counted by plating 100 µl decontaminated sputum onto 7H11 Middlebrook agar plates (BD Biosciences). The number of bacteria recovered from the MPN assay was determined as previously described[13].

Contamination controls were done by incubating 1 ml liquid aliquots of both the EPCFE mixture and the 7H9 + PANTA, as well as plating these on 7H11 agar plates and incubating them for 8 weeks at 37°C. Potential growth from the MPN assay was confirmed by plating any growth on blood agar as well checking for acid fast bacilli through smear microscopy of each positive well as described above (Figure S1).

Viable PCR using PEMAX dye

Sputa was collected for n=20 of the patients (n=10 routine, n=10 intervention) over the course of treatment (182 time points). Sputa were decontaminated as above with the exception that 2.7 % NALC and 0.5% trisodium citrate was used for decontamination as described previously [24] and the pellet was resuspended in 2 ml PB. Samples were prepared for testing using the PEMAX reagent monodose (GenIUL, Spain) by aliquoting 500 µl each into a control tube (no PEMAX) and a tube containing the PEMAX dye (50nM). These were then mixed via pipetting and incubated at 37°C in the dark prior to photo-activation using the PhAST Blue system (15 min, 100%) (GenIUL, Spain). Xpert sample reagent (SR) was then added to the tubes to a total of 2 ml, incubated for 15 min at RT and tested with Xpert as per manufacturer's instructions.

Statistical analysis

Data were analysed using GraphPad Prism (version 8.0.1). The D'Agostino & Pearson normality test was used to determine distribution of data and all paired analysis was done using the Wilcoxon matched-pairs signed rank t-test. Comparisons of results across arms were done using the Mann Whitney t-test. Comparisons of proportions were calculated using Stata (version 13.0) using the test of proportions. A p-values ≤ 0.05 was considered statistically significant.

Results

Patient information

There were 21 patients enrolled in the routine treatment arm and 22 in the intervention arm of the parent study. There were no significant differences in number of unfavourable outcomes between treatment arms (p=0.39). Of the 21 patients enrolled on the routine treatment arm, 7/21 (33%) had unfavourable outcomes [1/7 (14%) lost to follow up, 2/7 (29%) failed treatment, 4/7 (57%) were lost to follow-up]. Of the patients enrolled on the intervention arm 5/22 (23%) had unfavourable outcomes [1/5 (20%) failed treatment, 3/5 (60%) were loss to follow-up and 1/5 (20%) died – cause of death was TB meningitis]. A detailed report of the parent study's clinical outcomes is under preparation by the NExT study leads.

Routine microbiology

Smear and culture

At week 0, in the routine arm, 9/20 (45%) and 11/21 (52%) of patients were smear (ZN) and culture-positive respectively (Table 1). This declined through treatment with most patients converting to negative by week 10 of treatment [2/13 (15 %; p=0.07) and 3/17 (18%; p=0.02) smear and culture respectively] (Figure 3A). All patients were smear and culture negative after week 16 of treatment. In the intervention arm, 8/21 (38%) and 17/22 (77%) of patients were smear and culture-positive at week 0 (Table 1). This declined steadily: at week 10 having 2/21 (10%; p=0.02) smear positivity and 2/22 (5%; p=0.0001) culture positivity smear and culture positivity respectively with all patients converting to negative results after week 16 (Figure 3B). There were no significant differences seen in the proportion of positive tests throughout treatment between arms (Table 1).

Xpert MTB/RIF

Xpert results had a higher proportion of positive results, especially later into treatment, compared to smear and culture. For example at week 0, culture positivity was 12/21 (57%) and

18/22 (77%) for routine and intervention arms respectively, while Xpert was 8/10 (80%) and 6/10 (60%) and at week 8 culture positivity was 5/16 (31%) and 4/20 (20%) respectively and Xpert was 68 (75%; p=0.04) and 7/9 (78%; p=0.003) respectively. Xpert-positive results at week 0 were 8/10 (80%) and 6/10 (60%) for the routine and intervention arm, respectively (Table 1, Figure 3). This remained stable until week 10 where it declined to 4/8 (50%) in the routine arm and converted to negative results after week 40 (Figure 3A). In the intervention arm, the proportion of positive tests stayed similar until week 24 with 1/9 (11%) being positive after which all convert to negative results (Figure 3B).

Novel methods for treatment monitoring

Most Probable Number (MPN) Assay

There was no significant difference in the proportion of positive patients between routine arm and intervention arm for the first 24 weeks since treatment initiation (Table 1), nor between the MPN readouts with 7H9+PANTA (7H9 MPN) or the modified MPN assay with added EPCFE (EPCFE MPN) within each arm. With the exception of week 0 for both arms [median (IQR) MPN number: 1.8 (0.0-5.6) vs 3.5 (1.9-4.3) for 7H9 vs. EPCFE; p=0.005 for routine arm, and 3.3 (1.3–7.9) vs. 4.8 (2.8-8.4); p=0.04 for intervention arm], and week 1 of routine arm [3.5 (1.4-8.3) vs. 3.9 (1.4-8.7); p=0.03] suggesting a mixed-population of DCTB and culturable bacilli prior to week 0.

Routine arm: Across time points, the routine arm had 33/200 (17%) positive results for the 7H9 MPN assay and 46/200 (23%) positive EPCFE MPN assay results (p=0.11) respectively. There were 6/20 (30%) positive results at week 0 for the 7H9 MPN assay and 11/20 (55%; p=0.11) for the EPFCE-MPN assay and time to negativity was longer than for standard culture and smear, where positivity was still seen up to week 40 (Figure 4A). At week 24, 1/13 (8%) were positive for EPCFE only. There were 3 cases of MPN positives late into treatment at week 40 where 2/8 (25%) were EPCFE-positive and 1/8 (13%) 7H9-positive, and at week 84 where 1/4

(25%) were EPCFE -and 7H9-positive (Figure 5A, S2). These were all negative by conventional methods (smear and culture).

Intervention arm: The intervention arm had 34/178 (19%) and 44/178 (25%) were positive results for 7H9 MPN and EPCFE MPN (p=0.24) respectively across the time points. There were 6/19 (32%) and 8/19 (42%;p=0.50) patients positive at week 0 for the 7H9- and EPCFE MPN assay respectively which declined steadily through treatment. EPCFE had a slightly higher proportion of positive results than 7H9 MPN and MGIT 960 after week 6 (Figure 3A and B). At week 24 (the final week of treatment in the intervention arm) there were 1/17 (6%) positive for the EPCFE MPN assay only (Figure 5B). An additional specimen was collected for one of the patients at week 26, as they were showing clinical signs of treatment failure, and this was positive for both 7H9 and EPCFE MPN assays (Figure 5B, S1).

Patients MPN-positive in both arms and negative by conventional diagnostics

There were several instances across all time points [14/47 (30%) and 9/41 (22%) (p=0.40), for routine and intervention arms respectively], where only EPFCE detected positive growth and was missed by 7H9 MPN methods. Of these 10/14 (71%) and 5/9 (56%) were missed by MGIT960 sputum culture (Figure 5A and B) and, 2/8 (25%) and 2/6 (33%) were also missed by Xpert (and vPCR-Xpert; some Xpert data were not available). Furthermore, even though there were no significant differences seen between total bacilli quantified with either EPCFE and 7H9 MPN for most time points, ΔlogMPN data across all time points shows that EPFCE-positive samples contained more total bacilli than those detected by 7H9 MPN methods for both routine [median (IQR): 1.9 (1.4-3.9) vs. 1.6 (0.0-2.8) for EPCFE and 7H9 MPN respectively; p=0.0001] and interventions arms [2.4 (1.7-4.6) vs. 1.7 (0.8-3.4) for EPCFE and 7H9 MPN respectively; p=0.0001] (though no significance differences where seen between arms for either MPN method) (Figure 6A and B).

Viable PCR - modified nucleic acid amplification testing

Routine arm: Of the patients that had vPCR results, the routine arm had 8/10 (80%) and 9/10 (90%) positive results for standard Xpert and modified vPCR-Xpert at week 0 respectively (Figure 4A, Table 1). At week 40, 1/5 (20%) of patients were positive for Xpert and 0/5 (0%) for the vPCR Xpert, suggesting possible false-positive Xpert result, however similar results were seen at week 1 and 24 (all of which had "very low" semi-quantitation results), which may be due to variable sampling and sensitivity of Xpert at low bacillary load. After W65 all patients were negative (Figure 4A). Rates of vPCR-Xpert positivity were similar to Xpert and both had higher proportion positive results compared culture methods (Figure 4A, S3). The differences between C_{Tmin} values (ΔC_{Tmin}) did not differ significantly between Xpert and vPCR-Xpert throughout treatment (Figure 7A).

Intervention arm: There were 6/10 (60%) and 8/10 (80%) positive for Xpert and vPCR-Xpert respectively at week 0 (Table 1). At week 24, 1/9 (11%) and 2/9 (22%) were positive for Xpert and vPCR-Xpert respectively (Figure 4B). An equal number (four) of patients were positive only for Xpert or vPCR throughout treatment (all but one had "very low" semi-quantitation, data not shown). As with the routine arm, ΔC_{Tmin} values did not differ significantly between Xpert and vPCR-Xpert throughout treatment (Figure 7B) except at week 1 [median (IQR): 18.4 (17.1-23.4) vs. 17.0 (14.1-21.2); p=0.02] and at week 4 [22.7 (21.4-27.6) vs. 19.0 (16.8-24.7); p=0.02] of treatment, which may be due to sample size. All patients for which we had follow up had negative smear and cultures at post-treatment month 6 and 12.

Discussion

Previous studies have shown that modifying current, widely available diagnostics may be useful to increase their treatment monitoring potential. In this study, we looked at improving both standard culture and Xpert testing. Our key findings are: 1) including exponential phase cell-free extract to increase detection of differentially culturable bacteria did identify positive growth that standard culture (and Xpert) missed throughout treatment, 2) when positive for both standard 7H9 MPN and EPCFE MPN there were significantly more quantifiable bacilli detected with the EPCFE method, thus indicating DCTB are present through MDR-TB treatment, and 3) including a PMA/EMA (PEMAX) dye to sputa for Xpert testing did not significantly improve results.

While previous studies have shown that DCTB can be found in large numbers through treatment, including late in treatment, when cultured in the presence of EPCFE or RPFs [13,16], we found that although there were patients positive through treatment, the numbers were smaller than previously reported [13,14]. Though there were both differentially culturable -and standard culturable bacilli present in most positive samples, a larger percentage was detected using the EPFCE culture method, indicating that there are indeed DCTB present through MDR-TB treatment. Furthermore, there were several instances where the sample was only culture-positive using the EPFCE method and these patients would have been missed by standard culture otherwise. Of these, for which we had results, EPCFE detected 25% and 33%, as well as 71% and 55% for the routine and intervention arms respectively, which was missed by both Xpert and MGIT960 sputum culture. However, even if there were Xpert-positive results for those detected only by EPCFE (and culture negative), these results should be queried as they may be picking up DNA from dead cells due to TB treatment.

Previous studies have highlighted the fact that the addition of a dye such as PMA or EMA may reduce the detection of non-viable bacilli [22,24,25], including increasing specificity for TB

detection of viable bacilli using the Xpert MTB/RIF assay [27]. In this cohort we found that while there were differences between the C_{Tmin} values, these were not significant, including later into treatment, and while there were some patients that had positive results for standard Xpert only and not the vPCR, the converse is also true. We suspect this is due to several factors, one being that the microbiome in sputa likely interferes with the ability of the dye to bind to the TB bacteria that are present at lower numbers and that the mycobacterium cell wall is much harder to penetrate than other bacteria due to the mycolic lipid layer [28] .

Interesting to note, when looking at the early bactericidal activity (EBA) of the different regimens (especially with combinations of drugs used), the rankings would have first-line treatment where RIF and INH are combined, followed by BDQ and PZA containing MDR-TB regimens and then the FQ and SLID MDR-TB regimen. Though there were limitations in our study, this pattern showed in our findings where those with MDR-TB were more likely to be positive for any of the test if they were on the then-standard-of-care arm (FQs and SLIDs) versus the BDQ containing arm. However, it is possible that EBAs may differ in aerosol compared to sputum and this warrants further investigation.

The findings of this study should be interpreted with the limitations. Firstly, the inclusion criteria for the parent study changed after study initiation to include patients that had already been on two weeks of prior treatment, possibly affecting the amount of culturable bacteria in sputa. This may explain the presence of DCTB in sputa at week 0. Secondly, the criteria for treating people with DR-TB changed in South Africa during the study to exclude second-line injectables from the regimen and instead include bedaquiline, therefore the final total number of n=30 per arm was not reached, however we feel the sample size was big enough to elucidate preliminary findings. Finally we only tested patients from Cape Town area which may have introduced some bias in our findings as the Western Cape DR-TB epidemic differs to other regions in South Africa. DR-TB strains has been shown to be primarily atypical Beijing strains

found in the region, and these may react differently to the EPCFE as, for example the LAM/KZN strain found in the KwaZulu Natal region. Furthermore EPCFE from a different strain (not H37Rv) may also have different effects and should be tested.

We therefore conclude, that while methods for improving current diagnostics such as including EPCFE for detecting DCTB and vPCR dyes such as PEMAX for selecting only viable bacteria in sputa may have promise in some cases, for example detecting culture positives that may be missed by conventional methods, it did not significantly alter the outcomes of the patients. However, focus should be placed on coming up with new and innovative solutions to diagnostics for treatment monitoring, such as point-of-care biomarker tests or test that do not rely on sputum sampling. It may also be interesting to determine whether the DCTB correlate with PET-CT findings throughout treatment.

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Author contributions

A. E., K. D. and R. W. conceptualised the parent study, B. K. conceptualised the MPN methods and provided training in these methods. G. T. and R. V. conceptualised the sub-study. R. V., C. B., S. N. and B. D. performed experiments.

Conflicts of interests

The authors declare no competing interests.

Figures

Figure 1: [A] Overview of study timeline and **[B]** Sample collection and processing for this study. Patients were seen at each of the time points indicated and 3 sputa were collected on the same day. If only 2 sputa were collected they were used for culture and smear and MPN assay. Sputa were randomly used for either of the methods. Sputa for use in vPCR was decontaminated with N-acyl-L-cysteine (NALC) and sodium citrate rather than NaOH as this interferes with the dye. Abbreviations: ZN – Ziehl-Neelsen, MPN – most probable number, EPCFE – exponential cell free extract, vPCR- viable PCR, Xpert – Xpert MTB/RIF.

Figure 2: Set up of Most Probable Number (MPN) assay plate. The first three replicate rows (A- C; solid circles) contains 450 µl of a 1:1 exponential phase cell free extract (EPCFE) and fresh 7H9 broth with PANTA mixture. The second three replicate rows (D-F; dashed circles) contain 450 µl of only fresh 7H9 broth with PANTA for comparison. A 1:5 dilution series with decontaminated sputa was done across the plate as shown. Plates were scored weekly and kept for up to 8 weeks.

Figure 3: Percentage of positive tests including standard culture (MGIT 960) (squares, light-grey), smear microscopy (Ziehl-Neelsen staining) (diamonds, dark-grey) and standard Xpert (circles, black) on decontaminated sputa for both the **[A]** Routine arm where time-to-negativity (TTN) for smear and culture were 24 weeks for both and, **[B]** the Intervention arm where TTN for smear and culture were 16 and 24 weeks, respectively. Xpert had higher rates of positivity throughout treatment, possibly due to the detection of non-viable bacilli form TB treatment.

Figure 4: Xpert on decontaminated sputa (circles, solid line) versus modified vPCR-Xpert [where decontaminated sputa was pre-treated with a viability dye (PEMAX)] (circles, dotted line) has similar rates of positivity for and had higher rates than for MPN culture up until week 24 for both **[A]** routine arm and **[B]** intervention arm. EPCFE MPN (triangle, dotted line) had

slightly high rates of positivity than for 7H9 MPN (triangle, solid line) for both arms. Notably, for routine arm, MPN positivity was detected at week 40 and 84 where Xpert (and vPCR-Xpert and) were negative.

Figure 5: Patients who were positive for MPN in the **[A]** Routine treatment arm and the **[B]** Intervention treatment arm. There was no significant difference seen for EPCFE MPN vs 7H9 MPN throughout treatment (except at week 0 for both arms and week 1 of intervention arm). There were several instances where EPCFE detected growth that was missed by 7H9 MPN [14/47 (30%) and 9/41 (22%) for routine and intervention arms respectively. Red bars (left) indicate EPCFE supplemented growth and green bars (right) indicate growth in 7H9 broth only. The x-axis indicate logMPN (colony forming units/ ml). P-values were calculated using the Wilcoxon matched-pairs signed rank test.

Figure 6: Difference in logMPN values (ΔlogMPN) between EPCFE and 7H9 MPN results across all time points for all positive results for both **[A]** the routine arm and **[B]** the intervention arm across treatment. Though no significance was seen between total bacilli (logMPN) for EPCFE and 7H9 for most time points, significant difference were seen overall across all time points for both routine and intervention arms (though not between arms for either MPN method).

Figure 4: Difference in C_{Tmin} values **(**ΔC_{Tmin}) between those positive for both Xpert and vPCR-Xpert in the **[A]** routine arm and the **[B]** intervention arm across treatment. There was no significant difference detected between the Xpert and vPCR-Xpert samples. P-values of Xpert C_{Tmin} vs vPCR-Xpert C_{Tmin} per patients, per time point were calculated using the Wilcoxon matched-pairs signed rank test.

Figure 1

A

Routine arm: PZA, MOX, ETH, TRZ, KAN													Post treatment follow up	
Week	1	2	4	6	8	10	16	24	40	65	72	84	Month 6	Month 12
Intervention arm: BDQ, LZD LEV, PZA, INH, ETH, TRZ														
Week	1	2	4	6	8	10	16	24					Month 6	Month 12

B

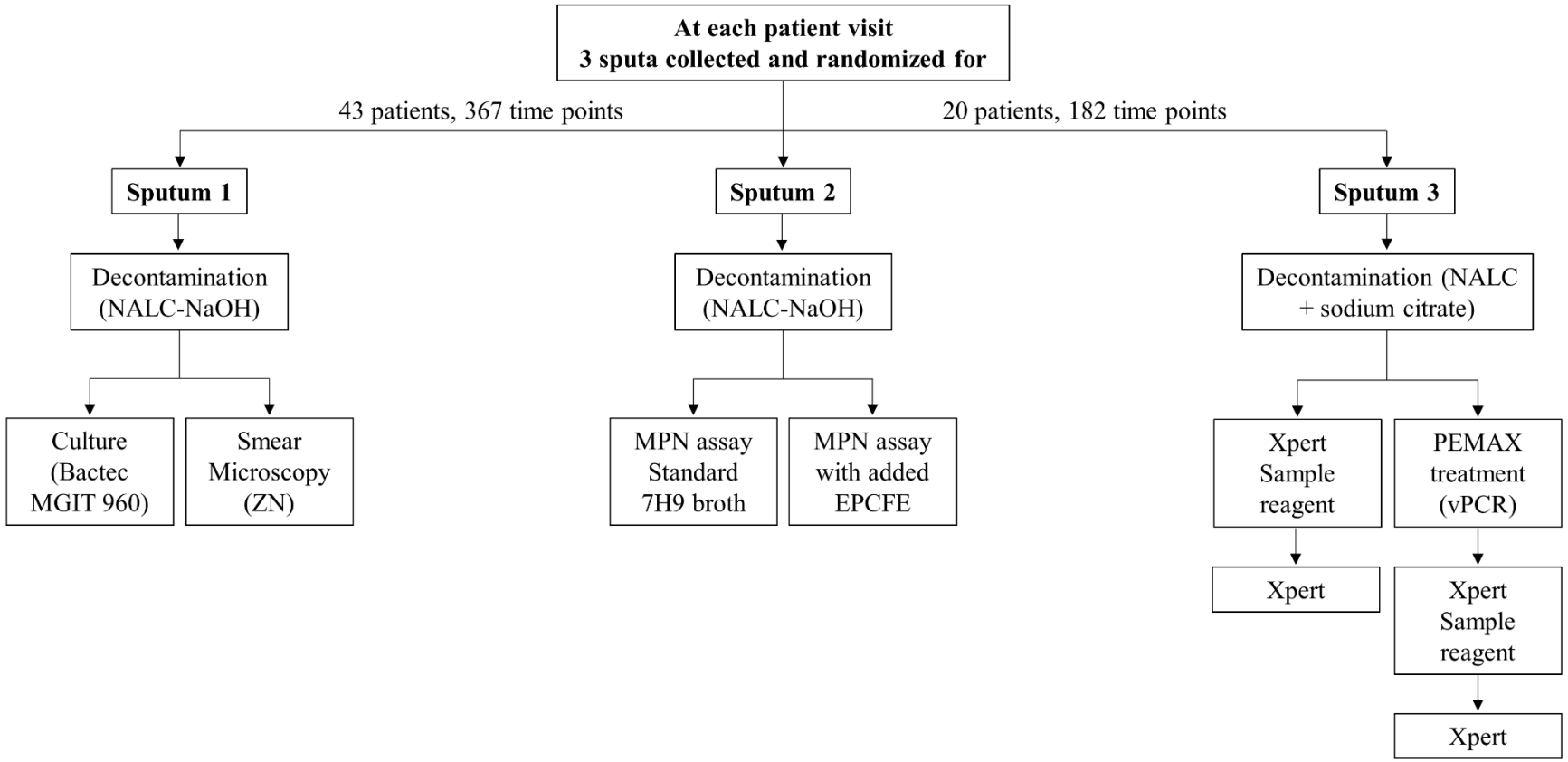

Figure 2

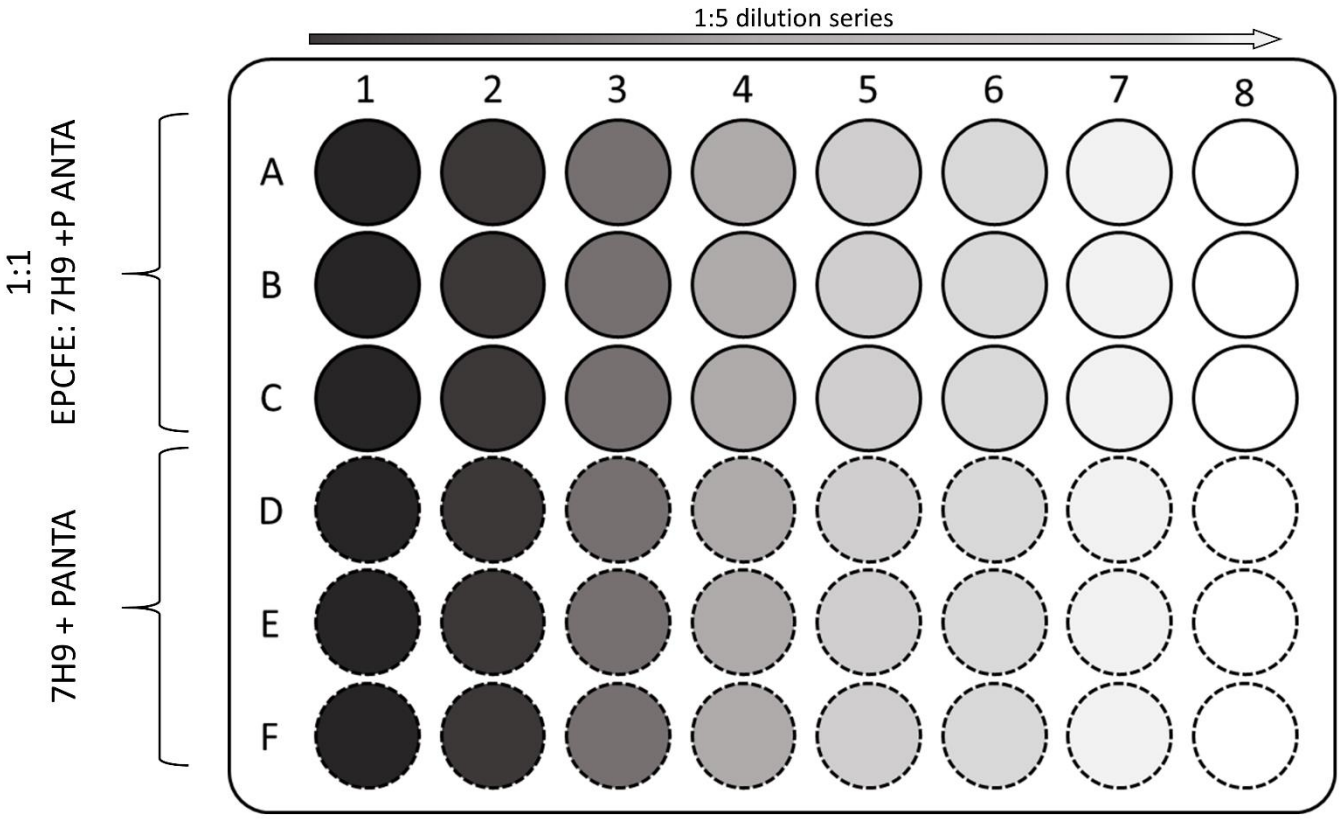

Figure 3

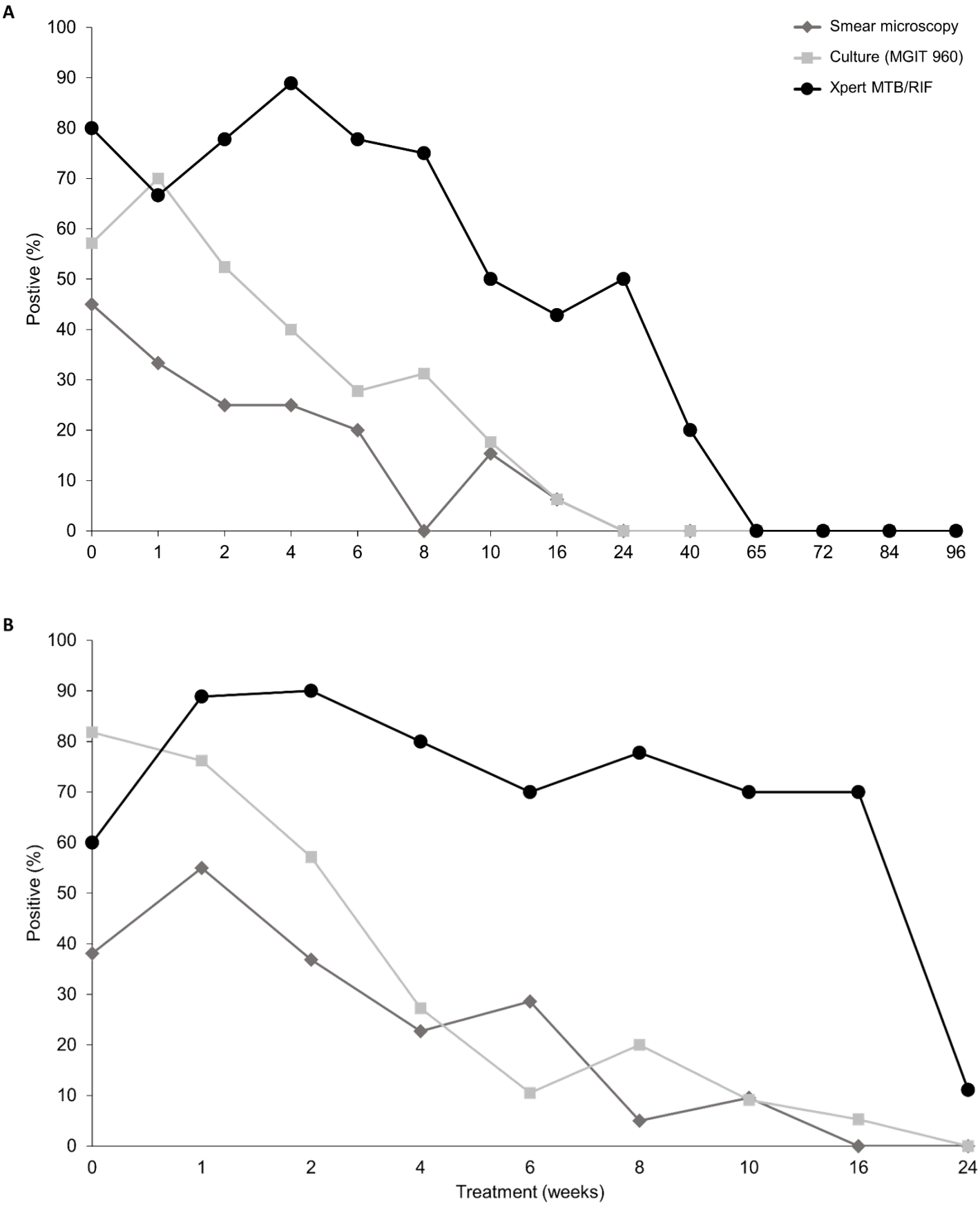

Figure 4

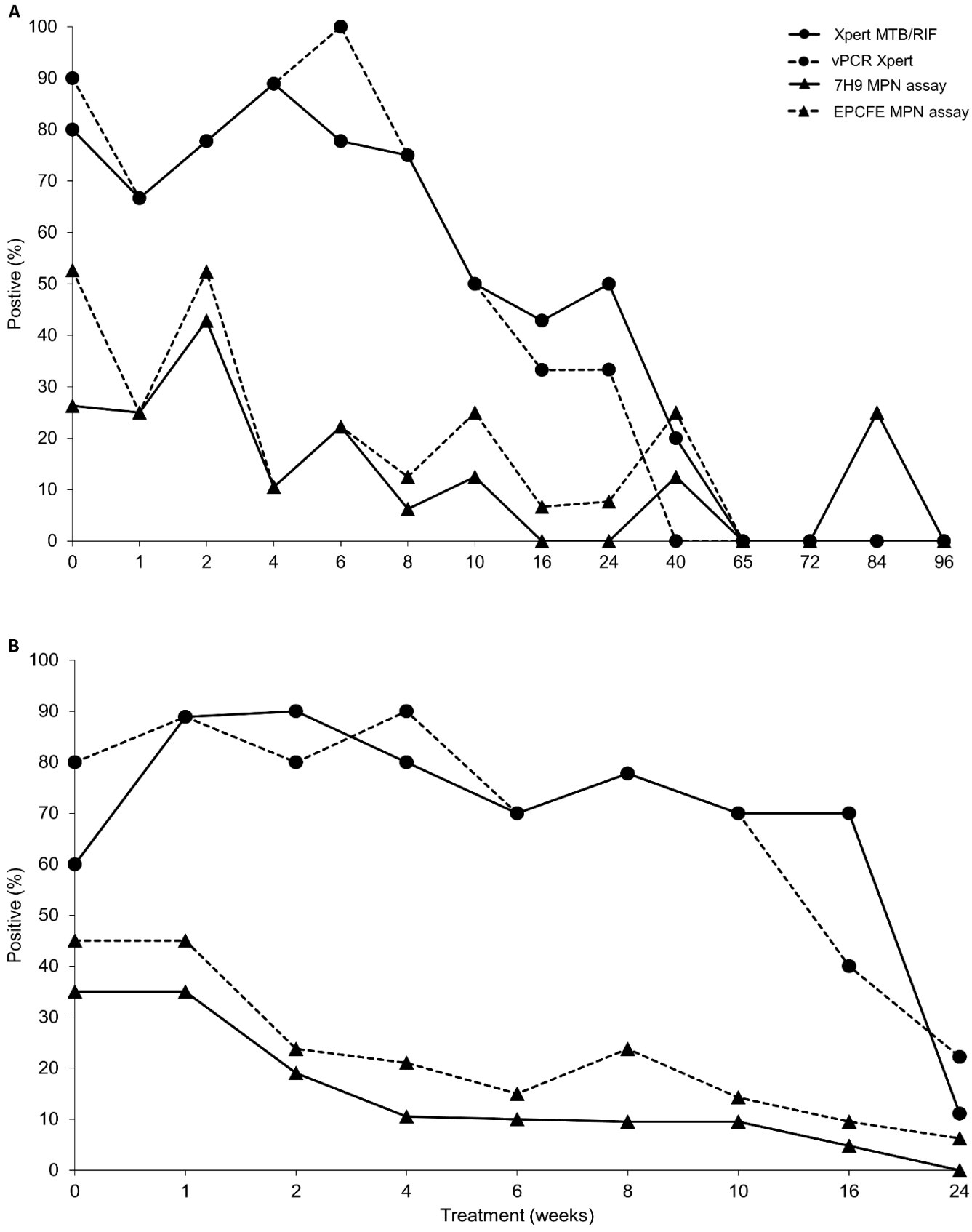

Figure 5

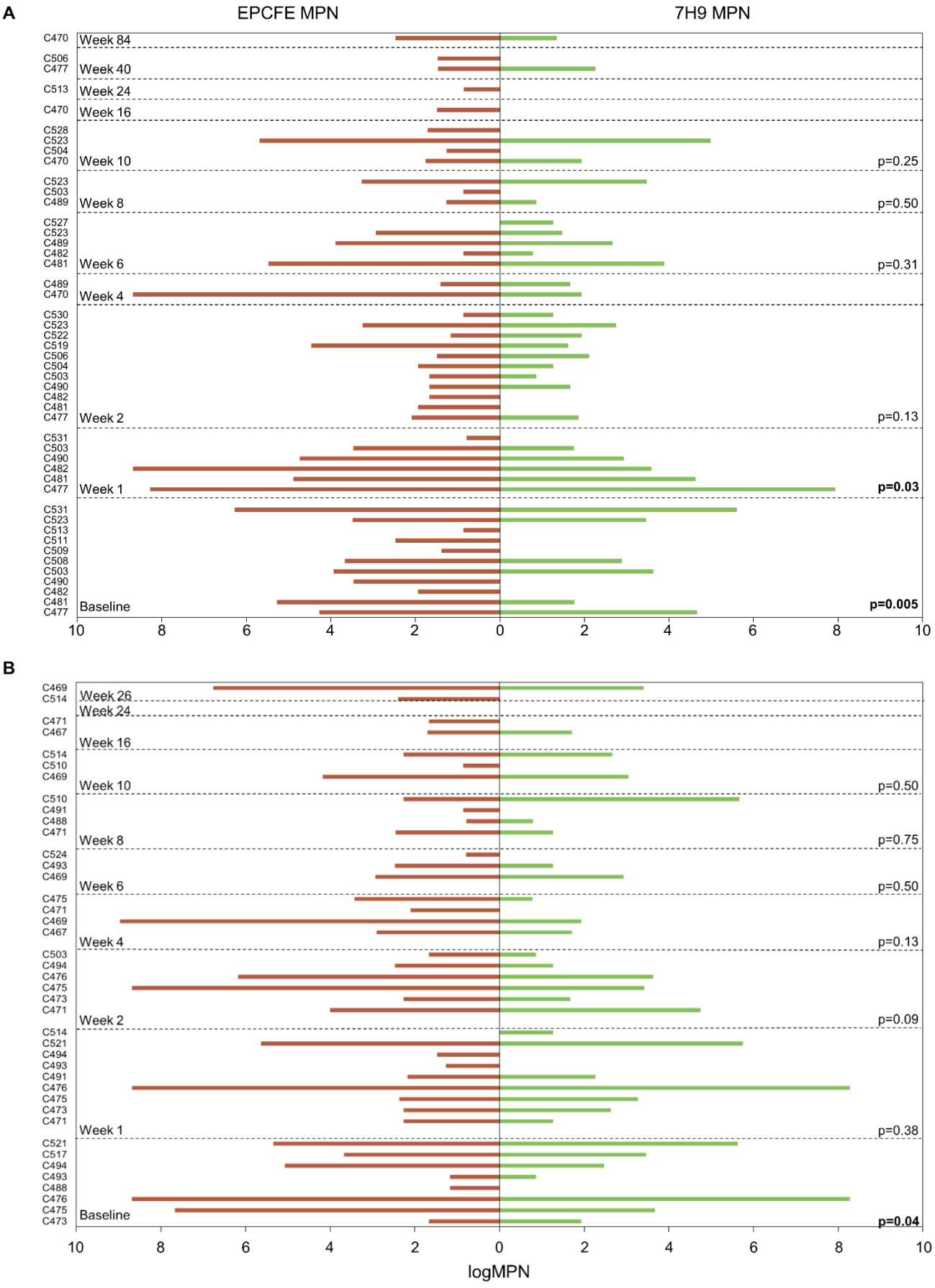

Figure 6

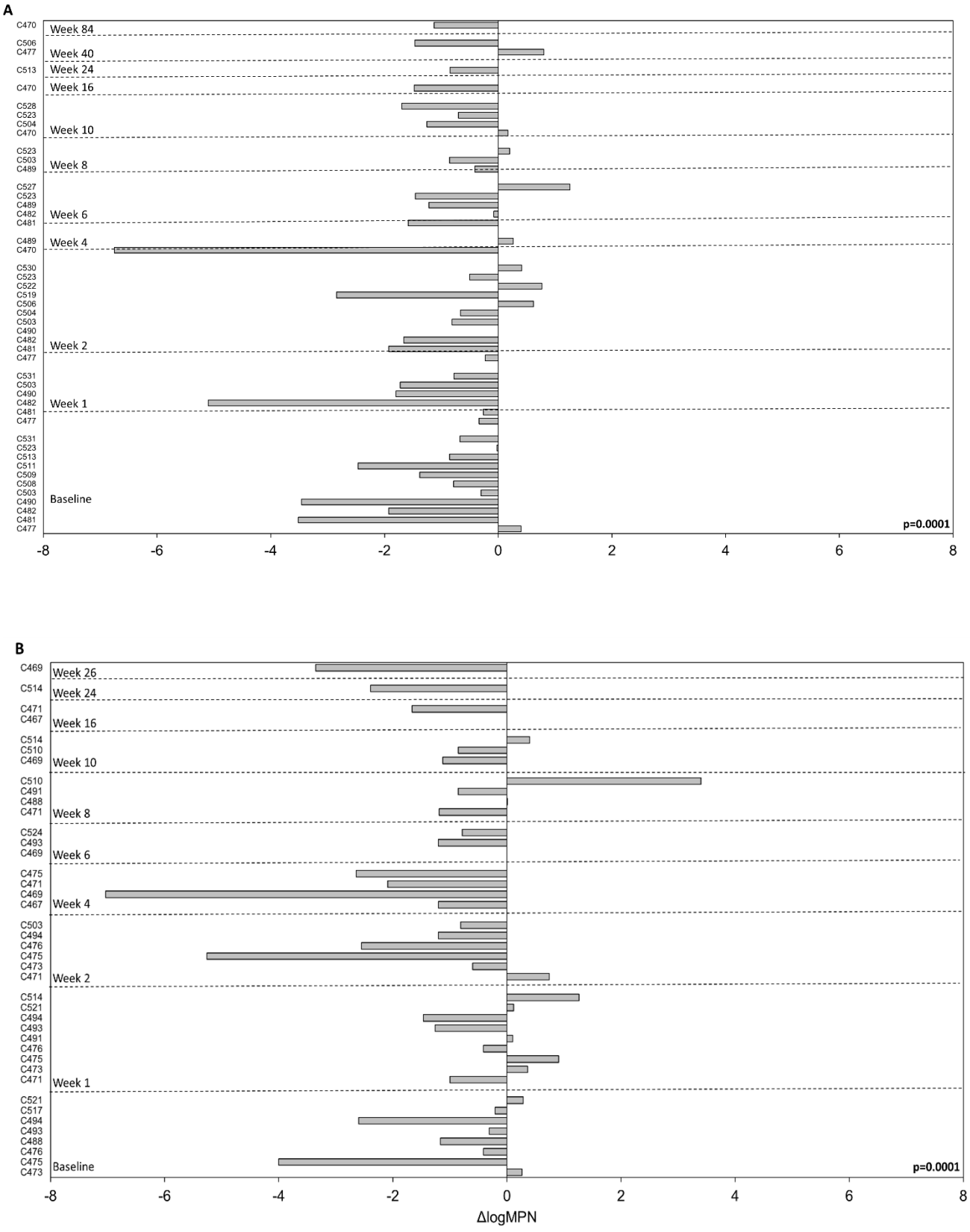

Figure 7

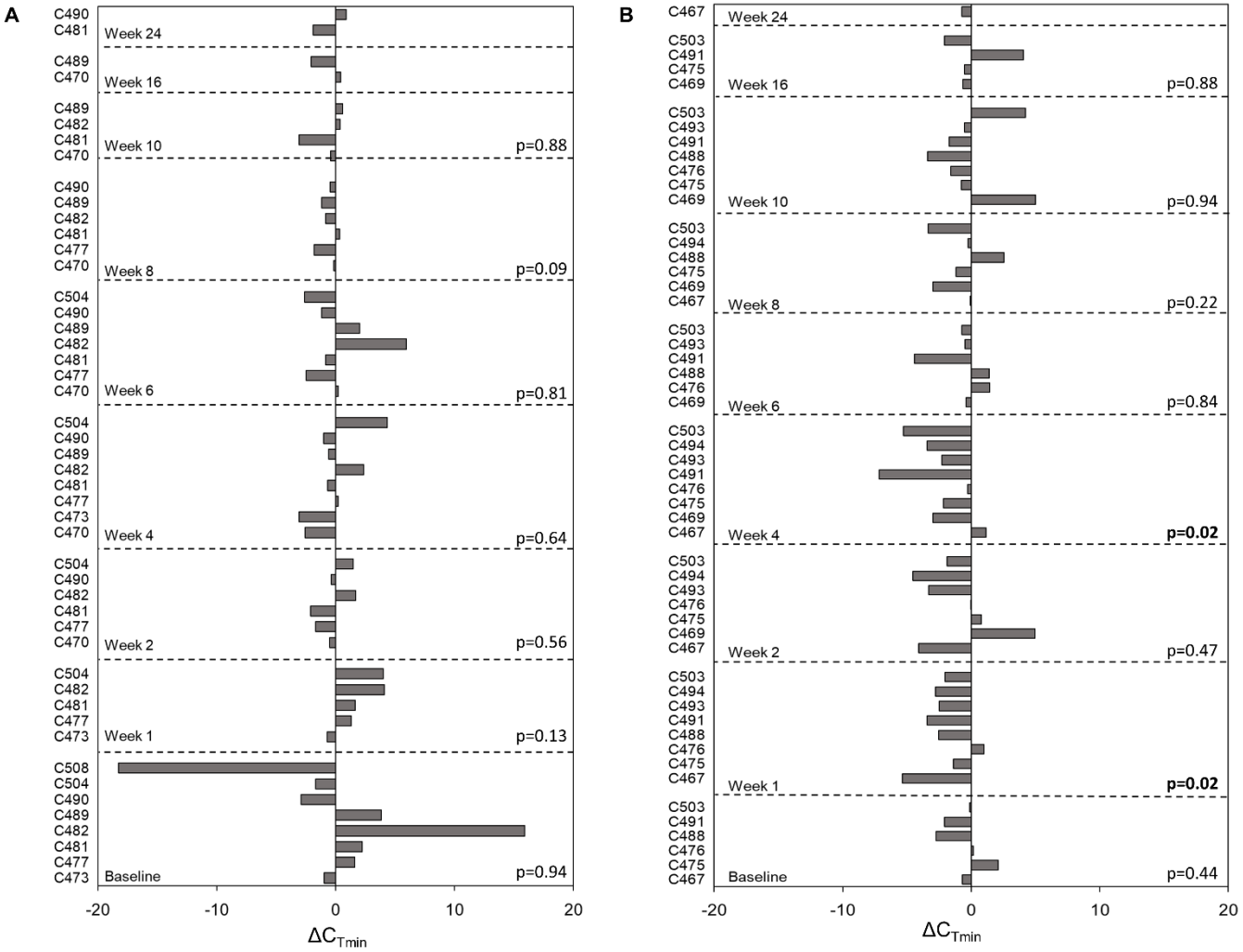

Table 1: Proportion of positive tests through the first 24 weeks of treatment for both routine and intervention arms. Proportion positives between arms were not significant by standard diagnostics nor modified diagnostic tests. *P-values for comparisons for a specific time point across arms. +P-values for comparisons between the modified and unmodified method within an arm for a specific time point. P-values represent proportion of positives. Data are n/N (%).

	Week																	
	0		1		2		4		6		8		10		16		24	
	R	I	R	I	R	I	R	I	R	I	R	I	R	I	R	I	R	I
Smear microscopy	9/20 (45)	8/21 (38) p=0.65*	6/18 (33)	11/20 (55) p=0.18*	5/20 (25)	7/19 (36) p=0.42*	5/20 (25)	5/22 (23) p=0.86*	3/15 (20)	6/21 (28) p=0.56*	0/17 (0)	1/20 (5) p=0.35*	2/13 (15)	2/21 (10) p=0.61*	1/16 (6)	0/19 (0) p=0.27*	0/15 (0)	0/19 (0)
Culture	12/21 (57)	18/22 (77) p=0.08*	14/20 (70)	16/21 (76) p=0.65*	11/21 (52)	12/21 (57) p=0.76*	8/20 (40)	6/22 (27) p=0.38*	5/18 (27)	2/19 (10) p=0.18*	5/16 (31)	4/20 (20) p=0.44*	3/17 (18)	2/21 (10) p=0.43*	1/16 (6)	0/19 (0) p=0.90*	0/15 (0)	0/19 (0)
MPN																		
7H9	5/19 (30)	7/20 (32) p=0.91*	5/20 (25)	7/20 (30) p=0.49*	9/21 (42)	4/21 (19) p=0.10*	2/19 (10)	2/19 (10) p=1.0*	4/18 (22)	2/20 (10) p=0.3*	1/16 (6)	2/21 (10) p=0.38*	4/16 (25)	3/21 (14) p=0.41*	1/15 (7)	2/21 (10) p=0.76*	0/13 (0)	1/16 (6) p=0.36*
EPCFE	10/19 (55) p=0.09+	9/20 (42) p=0.42* p=0.51+	5/20 (25)	9/20 (45) p=0.18* p=0.52+	11/21 (52) p=0.54+	5/21 (23) p=0.06* p=0.71+	2/19 (10)	4/19 (21) p=0.37*+	4/18 (22)	3/20 (15) p=0.57* p=0.63+	2/16 (13) p=0.54+	5/21 (24) p=0.72* p=0.21+	2/16 (13) p=0.37+	2/21 (10) p=0.77* p=0.63+	0/15 (0) p=0.31+	1/21 (5) p=0.39* p=0.55+	1/13 (8) p=0.31+	0/16 (0) p=0.26* p=0.31+
Xpert																		
Standard	8/10 (80)	6/10 (60) p=0.33*	6/9 (66)	8/9 (89) p=0.26*	7/9 (77)	9/10 (90) p=0.47*	8/9 (89)	8/10 (80) p=0.6*	7/9 (78)	7/10 (70) p=0.7*	6/8 (75)	7/9 (78) p=0.89*	4/8 (50)	7/10 (70) p=0.39*	3/7 (43)	7/10 (70) p=0.26*	3/6 (50)	1/9 (11) p=0.10*
vPCR	9/10 (90) p=0.53+	8/10 (80) p=0.53* p=0.33+	6/9 (66)	8/9 (89) p=0.26*	7/9 (77)	8/10 (80) p=0.91* p=0.53+	8/9 (89)	9/10 (90) p=0.94* p=0.53+	9/9 (100) p=0.14+	7/10 (70) p=0.07*	6/8 (75)	7/9 (78) p=0.89*	4/8 (50)	7/10 (70) p=0.26*	2/7 (29) p=0.56+	4/10 (40) p=0.63* p=0.18+	2/6 (33) p=0.56+	2/9 (22) p=0.63* p=0.53+

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Chapter 6

Use of a modified face mask and liquid-based cough aerosol sampling to measure the infectiousness of drug-susceptible and drug-resistant tuberculosis patients

Use of a modified face mask and liquid-based cough aerosol sampling to measure the infectiousness of drug-susceptible and drug-resistant tuberculosis patients

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Abstract

Background: Understanding when tuberculosis patients on treatment are non-infectious is critical, but current tools are suboptimal. To overcome these limitations, we did a pilot study to evaluate *Mycobacterium tuberculosis* detection in cough aerosol captured using a mask containing a gelatine filter (gMask; used for MGIT960 culture) or a liquid-based cough aerosol sampling system (LCASS) against the reference standard of the validated solid agar CASS.

Method: For gMasks, initial proof-of-concept and wearing-period optimisation studies (1, 3, 6 h) were done in 10 pre-treatment patients. We subsequently sampled 45 patients (some of which had started treatment) at least once for 1 h (130 total samplings). LCASS (containing two reservoirs) were done on a subset (n=14). Contents of each reservoir were randomly allocated to MGIT960 or the most probable number assay [with or without supplementation with exponential phase cell-free extract (EPCFE) to stimulate growth of differentially-culturable bacilli (DCTB)].

Results: For gMask sampling durations beyond 1 h, no increase in TB detection was observed. At the first visit (as close as possible to treatment initiation), CASS, gMask, and LCASS positivity rates were 6/44 (14%), 10/43 (23%) and 4/14 (29%) respectively. Of the 10 gMask-positives, three (30%) were sputum culture-negative. 1/28 (4%) of patients were CASS-positive one week after enrolment. gMasks had positive results until week 8 [8/82 (10%) gMask positive beyond 1 week]. LCASS readouts were not positive beyond baseline except for one patient EPCFE positive at week 8.

Conclusion: Methods for sampling cough aerosol that differ from the validated CASS approach can be used to identify different infectious patients. gMasks, which are more scalable, may be able to detect *Mtb* in aerosol in a more sensitive manner from patients on treatment. Finally, patients on treatment likely have bacilli in aerosol with a dormancy-associated phenotype.

Introduction

Drug-resistant (DR-) TB is a growing concern with 588 000 incident cases reported in 2017 [1]. Several house-hold contact and epidemiological studies have shown that person-to-person transmission is a major driver of DR-TB[2-6]. Evidence suggests patients on effective treatment have a decrease in their infectiousness after two weeks and convert to a smear-negative status. However, there while guinea pig models suggest that patients with DR-TB are no longer infectious when on treatment[7], there remains limited evidence of this for person-to-person transmission of DR-TB. This is needed to inform infection control in hospitals[8-10]. There is limited data on the infectiousness of DR-TB throughout treatment, including the new standard multi-drug resistant (MDR-) TB regimen which includes bedaquiline. This information is needed to inform on regimen strengthening and when patients can be safely discharged[11,12].

The Cough Aerosol Sampling System (CASS) is a technology used to quantify both the size and distribution of individual aerosol particles from TB patients using an Anderson Cascade Impactor (ACI). The ACI consists of six stages that holds solid agar plates. Each level contains specifically engineered holes which allow for particles of certain sizes to travel down through the impactor, the diameter of which decrease with each stage. The inertia and sizes of the holes allow particles that correspond to the different respirable aerosol ranges (bottom 4 stages) to be trapped on specific stages. CASS has been validated against transmission in humans and is therefore used as a proxy measure for infectiousness[12-15]. Though CASS is the only validated means of measuring infectiousness, it does have some limitations. Namely, it requires extensive infrastructure to run the machine and is dependent on a short period (5 min) of forced cough, rather than spontaneous coughing. Recent studies have shown the use of a modified face-mask containing a gelatine filter that traps aerosolised *Mycobacterium tuberculosis* (*Mtb*) particles which can be recovered from the solubilised gelatine filter. This has previously been tested on a small subset of TB-positive patients in the United Kingdom and Pretoria, South Africa[16,17] as

well as patients with chronic obstructive pulmonary disease (COPD)[18]. It has, however, not been tested on patients with DR-TB, especially in high-burden settings such as the Western Cape, nor has it been evaluated longitudinally on patients receiving MDR-TB treatment.

Another limitation is that CASS captures aerosols on solid media, while liquid have been shown to be more sensitive for culturing of TB and has the advantage of permitting rapid identification using molecular assays. Liquid culture is also potentially useful for studying the influence of novel compounds (e.g., resuscitation promoting factors - RPFs) on the recovery of differentially culturable *Mtb* (DCTB) from the cough aerosol[19,20]. Liquid aerosol sampling technology has not been used for TB previously but has been validated for other bacteria such as *Legionella spp*. and *Campylobacter spp.* as well as for the influenza virus[21-23].

We therefore aimed to design both a gelatine filter-containing mask (gMask) and a liquid cough aerosol sampling system (LCASS) to test on patients who are routinely undergoing CASS as part of ongoing studies, which will be used as a reference standard.

Materials and Methods

Study population

Patients enrolled at Brooklyn Chest Hospital (BCH) were invited to participate in the cough aerosol sampling (CASS) study. This study was approved by the Health Research Ethics Committee of Stellenbosch University (HREC Ref No: N13/01/001). A large proportion of the patients were enrolled in a larger funded clinical drug trial (NExT; Clinicaltrials.gov #NCT02454205) which evaluated a new MDR-TB regimen containing bedaquiline and linezolid (intervention arm; 6 month regimen) compared to the standard MDR-TB treatment (standard treatment arm containing fluoroquinolones and second-line injectables; 24 month regimen). For the CASS and gelatine mask (gMask) sub-study, an additional small proportion of pre-treatment DS-TB patients were enrolled from other sub-studies. Eligible persons were recruited from the Western Cape, South Africa from November 2015 to December 2018 (Figure 1A).

Cough Aerosol Sampling System (CASS)

An ACI that holds 7H11 Middlebrook (BD Biosciences, United States) agar plates with Mycobacterial SELECTA-TAB (Mast Group Ltd) was prepared fresh weekly, stored at 4°C, and placed in the CASS on the day of use (Figure 2A). The agar plates are removed and incubated at 37°C for 6 weeks and scored weekly for growth. Suspected TB colonies are tested via Ziehl-Neelsen staining[24]. The apparatus was set up at BCH in a specifically designed negative-pressure booth where the patient could cough into this apparatus for 5 min (Figure 2B). If a patient produced sputa during coughing, they were ask to deposit this into a collection jar which was used for routine culture and smear microscopy. Cough frequency was also measured.

Gelatine Masks (gMask)

Preliminary studies and optimal duration of wear

A N95 mask was chosen as this was routinely worn and sturdy enough to support the gelatine filter (gMask) (Figure 3A). The patients wearing the gMask were asked to cough spontaneously when they needed to. An initial study to determine feasibility of the gMask was tested on 10 treatment-naïve patients (Figure 1B). A further study was then done on 10 patients to determine the optimal duration time of wear for the gMask where a patient was asked to randomly wear the gMask for either 1 hour, 3 hours or 6 hours on consecutive days (Figure 1C). A survey was also conducted to determine cough frequency while the patient was wearing the gMask as well as the times the gMask was worn.

gMasks sampling through treatment

Following the preliminary results, patients enrolled for CASS were asked to wear the gMask for one hour on the same day. For a sub-set of patients, LCASS was also done on a different day (the day the patient would wear the gMask and whether CASS or LCASS were on the first or second day were randomised). Patients not enrolled in the clinical drug trial but that were enrolled in the CASS study did not get additional LCASS (Figure 1A).

The gMask was processed by removing the filter (Figure 3B) with sterile forceps and placing it in 5 ml N-acetyl-L-cysteine-sodium hydroxide (NALC-NaOH; 0.5 M NaOH, 0.37 g/L N-acetyl-L-cysteine and 0.05 M trisodium citrate dehydrate), using the commercial MycoPrep kit (BD Biosciences) to remove any contaminants. The sample was then neutralised by adding PB (33 mM Na_2HPO_4, 33 mM KH_2PO_4; pH 6.8) and the suspension centrifuged at max speed (4000 x g) for 15 min at RT. The pellet was resuspended in 500 µl 7H9 broth and used for MGIT culturing to determine if any culturable TB bacteria can be detected. Any leftover sample was stored at -20°C. Gelatine filters were used for culture, rather than molecular testing methods, as we wanted to determine which patients had culturable bacilli in their sputa.

Liquid Cough Aerosol Sampling System (LCASS)

LCASS, based on the CASS, was designed and manufactured (Figure 2C). It is set up in a similar fashion to the CASS with the pipe the patient coughs down connected to the first three stages of the ACI (containing 3 SELECTA-TAB 7H11 Middlebrook agar plates) which filters out larger particles and allows for the particles corresponding to the respirable range to pass through to 2 SKC BioSamplers (SKC Ltd, UK). One sampler contained phosphate buffer (PB) and the other 7H9 Middlebrook broth with added Oleic Albumin Dextrose Catalase (OADC, BD Biosciences). Sampling was done as described for the CASS. The agar plates were incubated at 37°C for 6 weeks and scored weekly for growth. The liquid from both chambers were carefully removed, centrifuged at max speed (4000 x g) for 20 min at room temperature (RT) and the pellets resuspended in 500 µl PB. All samples from the PB-containing SKC sampler and the 7H9-containing SKC sampler were randomly used for either BACTEC MGIT 960 (BD Biosciences) culturing or the Most Probable Number (MPN) assay using standard 7H9 broth and exponential-phase cell free extract (EPCFE) from growing H37Rv TB cultures to promote growth of DCTB as previously described[20]. CASS and LCASS sampling occurred within 48 hours of each other to give the patient resting time between sampling events. The order of sampling was randomized. A patient was seen at first sampling point (week 0), week 1, week 2 and week 4. If the patient had a positive CASS or LCASS at week 4, they were asked to return for further sampling.

Culture and smear

Sputa were decontaminated using the NALC-NaOH method as described above. Briefly, an equal volume of NALC-NaOH was added to the sample and vortexed, after a 15 min incubation at RT, specimens were neutralised with PB and centrifuged at max speed (4000 x g) for 15 min at RT. Pellets were resuspended in 1.5 ml PB, 500 µl was used for liquid culture inoculation using the BACTEC MGIT 960 system (BD Biosciences) supplemented with 800 µl PANTA

(containing a lyophilized antibiotic mixture of polymyxin B, amphotericin B, nalidixic acid, trimethoprim and azlocillin; BD Biosciences). After ~30 µl was used for smear microscopy using Ziehl-Neelsen staining technique (acid fast stain)[24], the rest of the specimen was biobanked at -20°C.

Biosafety methods for collection and transport of samplers

Aerosol sampling for the CASS and LCASS were done in a negative pressure booth that was fitted with HEPA filters and UV lights hidden in sconce to protect patients. All study personnel also wore personal protective equipment (masks, gloves, surgical gowns, shoe covers). gMasks were placed in double zip-lock bags and a sealed container for transportation. CASS and LCASS were likewise sealed in double layers of auto-clave bags, sealed with zip-ties and placed in sealed containers for transportation. All the above were processed in a Biosafety level 3 laboratory.

Results

Cough Aerosol Sampling System

A total of 132 time points were sampled across 45 patients and of these time points 7/132 (5%) were CASS positive [6/44 (14%) positive at week 0, 1/28 (4%) was positive at week 1] (Table 1, Figure 4). Of the 6 week 0 positives all but one were pre-treatment DS-TB patients. The remaining patient had DR-TB and was enrolled in the intervention arm (C510, they were also CASS positive at week 1) (Table 3).

Gelatine Masks

Preliminary testing

Of the initial 10 patients tested, 2/10 (20%) were gMask aerosol culture-positive (gMask-positive) and one was contaminated. (Table 1). Of the gMask-positive patients 1/2 (50%) were CASS-positive and the other CASS-negative.

Optimal duration times

Of the patients in the duration study, 4/10 (40%) gMask-positive for all 3 time points (with the exception of one of the four – who only had 1 positive time point) (Table 1). The burden of bacterial load [using time to positivity (TTP) as a proxy measure] did not differ significantly between the duration of wear ($p=0.47$). One hour was chosen as sufficient time to continue with future testing as there was no significance seen between the bacillary loads recovered from the aerosol of those positive at different time points. Of the gMask-positive patients 2/4 (50%) were CASS positive and of the remaining six patients that were gMask-negative, 2/6 (33%) were CASS-positive, indicating gMask may miss patients CASS detects and vice versa (Table S1).

Longitudinal testing

gMasks were done at 130 time points across 45 patients. A total of 22/130 (17%) were gMask-positive using the MGIT 960 system across all time points of which 16/22 (73%) were sputum-

culture positive. At week 0, there were 10/43 (23%) gMask-positive patients and positive gMasks were detected at each time point up until week 8 [1/11 (9%)]. At week 10 all gMasks tested were negative (Table 1).

One patient was gMask-positive at only weeks 4 and 6 [this patient defaulted treatment at 6 months (routine arm)] (Table 3). One patient was gMask positive at week 1 and 8 of treatment (this person was also LCASS-positive for that week – intervention arm), this patient was also asked to provide an additional sputum at week 28 of treatment as they were showing clinical signs of treatment failure, which may be why they were aerosol-positive at week 8 (their treatment was extended for an additional four weeks, at which point they were culture-negative).

Liquid Cough Aerosol Sampling System

A subset of 14 were also sampled across a total of 54 time points with the LCASS. Of these, 4/14 (29%) were positive only at week 0 and two of the four were on the routine arm (Table 1). One of these patients (C364) were positive for LCASS, gMasks and CASS and were recorded as being non-adherent. The remaining patient was enrolled in the intervention arm and was also LCASS-positive at week 8 of treatment in the intervention arm using the MPN assay with EPCFE included to promote growth of DCTB (this person was also gMask positive at week 8)

Cough aerosol sampling comparison across methods

At week 0, of the 10 gMask-positive patients, 2 were also positive for CASS alone – both of which were pre-treatment patients not enrolled in the longitudinal drug trial (Table 3). One was also positive for LCASS (routine DR-TB treatment arm). Finally, one patient was also positive for both CASS and LCASS, and who was recorded as non-adherent. Of the 7 CASS-positives at week 0, two were also positive for gMask and one was also positive for both gMask and LCASS as mentioned above (the other three were gMask contaminated, and one was pre-

treatment for DS-TB). There were no patients that were positive for both CASS and LCASS only. Of four LCASS-positives, two were on the routine arm and one on the intervention arm (Table 3). The patient that was on the intervention treatment arm that was LCASS-positive, was also LCASS-positive at week 8 using the MPN assay which selects for DCTB as mentioned above. At week 1 of treatment there were no patients that were positive for at least two of the methods (Figure 4). Patients were more likely to be positive for any method if they were on the routine arm (20/28 vs 8/28; p=0.001). Of those who were positive for the intervention arm 3/4 (75%) either showed signs of clinical treatment failure late into treatment and had their regimens extended or had an unfavourable outcome.

Sputum culture and smear

At week 0, 31/45 (69%) of patients were sputum culture-positive and 23/27 (62%) had positive smear microscopy results (Table 2). Of the 10 aerosol gMask-positives at week 0, seven (70%) were also sputum culture-positive (including 2 CASS-positives, one of which was the patient that positive for all 3 methods). Interestingly, the one patient that was gMask -and LCASS-positive at week 0 was culture negative. All patients CASS-positive at week 0 were also positive for both sputum culture and smear. Of the LCASS-positive patients at week 0, 2/4 (50%) were sputum culture-positive, and only one was smear positive (C364, the patient that was positive for all 3 methods). At week 1, 2/4 (50%) of patients that were gMask positive were also sputum culture positive and the one positive CASS patient was also culture positive. The patient that was positive for both gMask and LCASS EPCFE-positive at week 8 was sputum culture-negative (but had been sputum culture-positive up to week 6).

Discussion

We have shown: 1) the gMasks are a non-invasive, mobile means to act as a potential measure of infectiousness and has higher rates of detection than either the CASS or LCASS, 2) 1 hr is sufficient for a patient to wear the gMask to detect aerosols and there was minimal additional yield from longer sampling durations, 3) gMasks suggests that some patients may remain infectious up to 2 months after treatment initiation, 4) that the novel LCASS system designed by our lab is comparable to that of the CASS for solid media and 5) using novel means of culturing such as adding EPCFE may help detect differentially culturable bacilli in aerosols and that a positive sputum culture does not also indicate when a patient may be infectious.

gMask has advantages in that is it mobile, small and easy to use, and relies on spontaneous rather than forced cough. It has also been shown to detect culturable bacilli later into treatment than either CASS or LCASS, however, this was a pilot study and more data are needed. The improved yield of gMasks. This may be due to the fact that patients are free to cough when they need and are not limited to testing in only a 5 min forced cough window. It may also be due to the fact that sputum spray is produced and is caught on the filter. However, we did ask patients to record when they coughed and encouraged patients to deposit any sputa produced into a collection cup after lifting the gMask. It also has the advantage that molecular testing (such as polymerase chain reactions to detect the presence of bacteria) can be done on bacteria that are actually aerosolized, captured in the filter and subsequently dissolved in media. Recently, a study from India also assessed whether RNA could be detected from bioaerosol from cellulose acetate masks or normal face masks thereby allowing detection of viable aerosols only, which is promising[25].

The LCASS provides an alternative measure of infectiousness to the solid agar method within the conventional CASS. This is useful for molecular techniques such as polymerase chain reaction (PCR) which could allow faster detection of aerosolised bacilli. As with the CASS, it

requires the necessary infrastructure to allow for safe and comfortable sampling. It is important to note that the gMask detected some patients as aerosol-positive while culture missed these patients.

The results of the study must be interpreted within its limitations. Firstly, it must be noted that the patients with DS-TB were sourced from various other case finding and house-hold contact studies and were all treatment naïve whereas those that had DR-TB were all sourced from the NExT study and received either the then-standard-of-care MDR-TB treatment or the new regimen containing bedaquiline. However, the enrolment criteria for the NExT study was altered to allow patients that have been on treatment for up to 4 weeks to enrol. This very likely had an effect on the number of patients that were able to produce detectable and culturable aerosols and may have biased the results presented here. However, it is still important to note that this study proves the promise of both the LCASS and gMasks and shows that they are likely at least as useful as the CASS. However, it must be noted that in the validation experiments, all patients that were enrolled were controlled for treatment status, and so we were confident in those findings.

In conclusion, it is clear that each approach has its advantages, but it would be useful to combine at least two of these for thorough aerosol testing, as each test missed patients that were picked up by the others. In the future, it would also be beneficial to test both gMask and LCASS using molecular methods in a systematic longitudinal manner starting with pre-treatment patients. We have generated the preliminary data to justify such an investigation.

Figures

Figure 1: Flow diagram showing **[A]** testing of gMasks and liquid cough aerosol sampling system (LCASS) against the cough aerosol sampling system (CASS) as a reference standard (grey boxes). Patients from ongoing studies that are routinely sampled with CASS were asked to also wear gMasks and/or LCASS sampling. Studies included a longitudinal drug trial measuring a new bedaquiline containing regimen for MDR-TB against the routine standard of care regimen as well as pre-treatment DS-TB patients. **[B]** Flow diagram showing protocol for preliminary feasibility study of gMask design. **[C]** A study was then designed to determine the optimal time for which the gMask can be worn and still give reasonable results.

Figure 2: [A] The Anderson Cascade Impactor – each stage has different diameter holes to allow particles through that lessen with size. Stages 4 onward correspond to particles within the respirable range (<5µm) [26]. **[B]** The CASS machine (with outer cover removed) that contains the Anderson Cascade Impactor. Patients sit in a negative pressure room and cough down a sterile pipe (with a disposable mouth piece which the patient puts their mouth around to cough) into the CASS (attached to a pump which draws the air through) for 5 min. **[C]** The set-up of a liquid sampling system which consists of an inlet for the pipe **(1)** where the patients cough (as for CASS) into a canister containing the first 3 stages of the Anderson Cascade Impactor **(2)** to trap the larger particles. The canister is connected to a cabinet containing two SKC BioSamplers with phosphate buffer or 7H9 inside to trap smaller aerosols **(3)** the whole system is connected to a pump that draws air through the apparatus (as for the CASS) and creates a vortex in the SKC Samplers **(4)**. **[D]** The inside set up of the LCASS. The two SKC samplers are placed in the cabinet and contain liquids as mentioned above. The inlet (that is connected to the canister) comes in at the top and air is drawn through the outlet on the side of the samplers thus creating a vortex.

Figure 3: Example of a gMask. **[A]** N-95 mask modified to contain aluminium holder containing gelatine filter. **[B]** A gMask that has been worn for 1 hour, the filter (which is now clear and jelly-like) is removed with sterile forceps, dissolved and decontaminated with NALC-NaOH before culture.

Figure 4: Venn-diagram showing the total number of positive cases for both CASS, LCASS and gMasks at week 0 and week 1 after treatment initiation. At week 0, three of the 10 gMasks were also positive for CASS, one was also positive for LCASS and one was positive for both LCASS and CASS. No CASS and LCASS were both positive while gMask was negative. At week 1, there were patients that were positive for at least 2 of the sampling methods.

Figure 1:

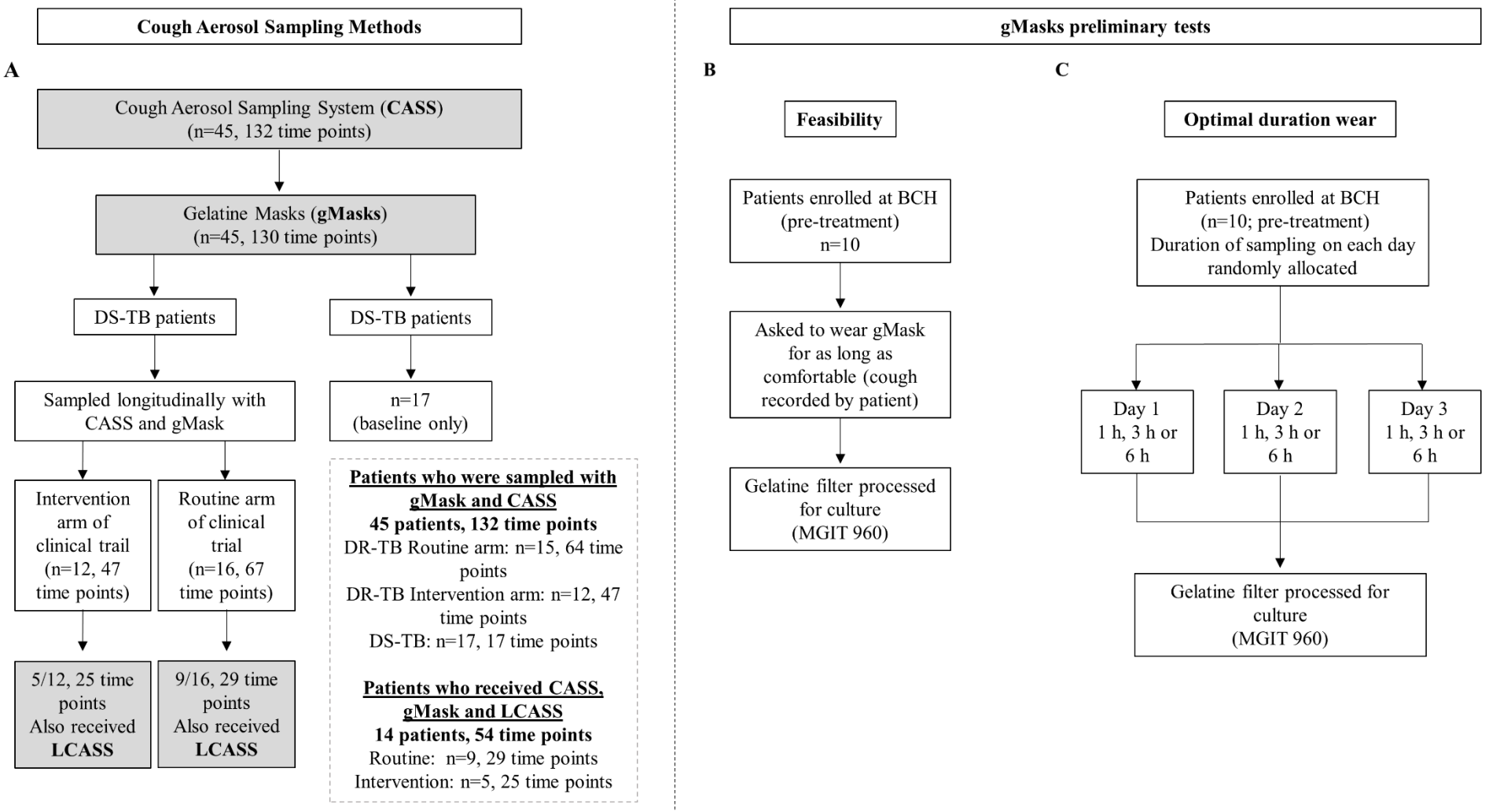


Figure 2

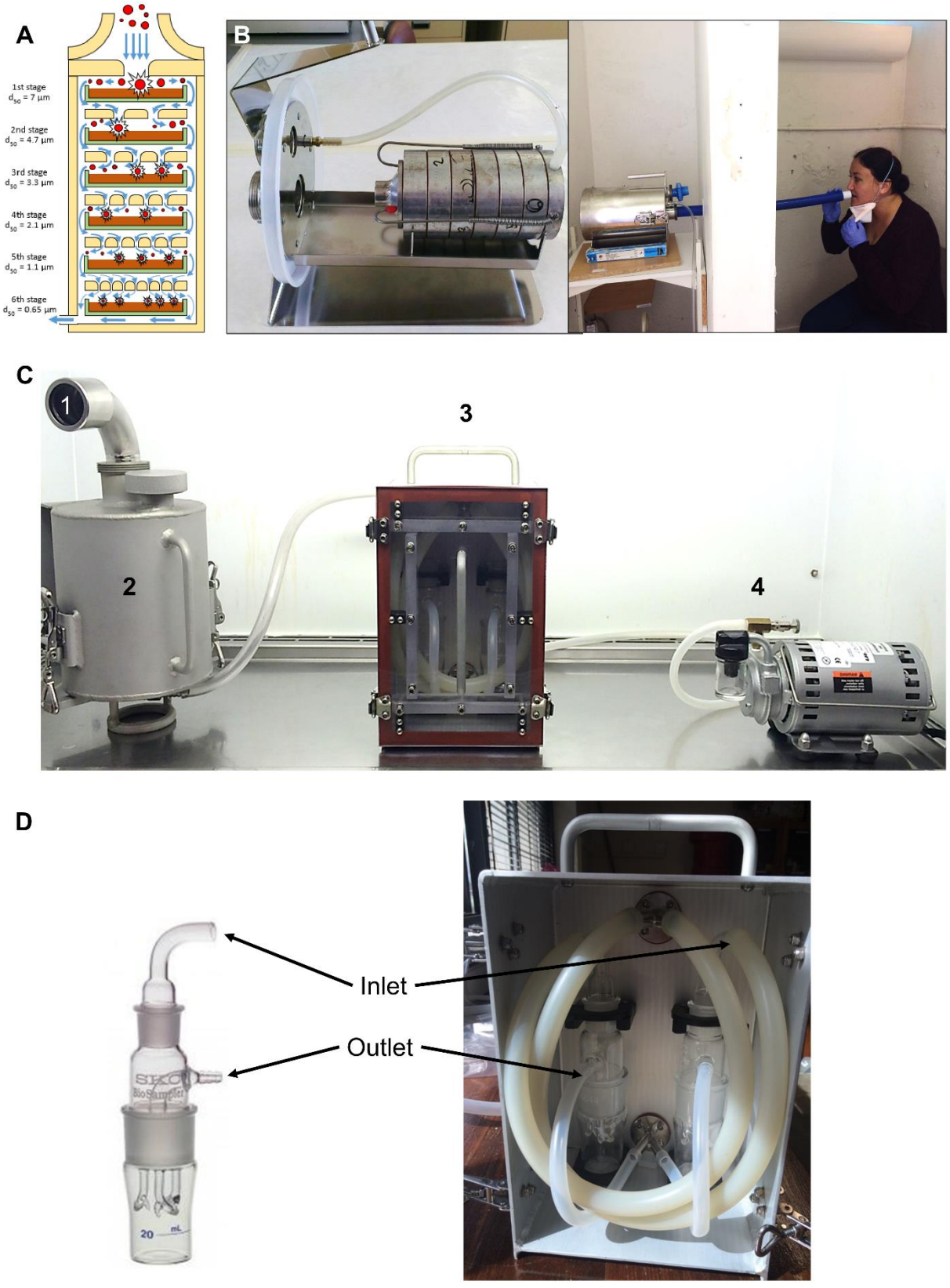


Figure 3

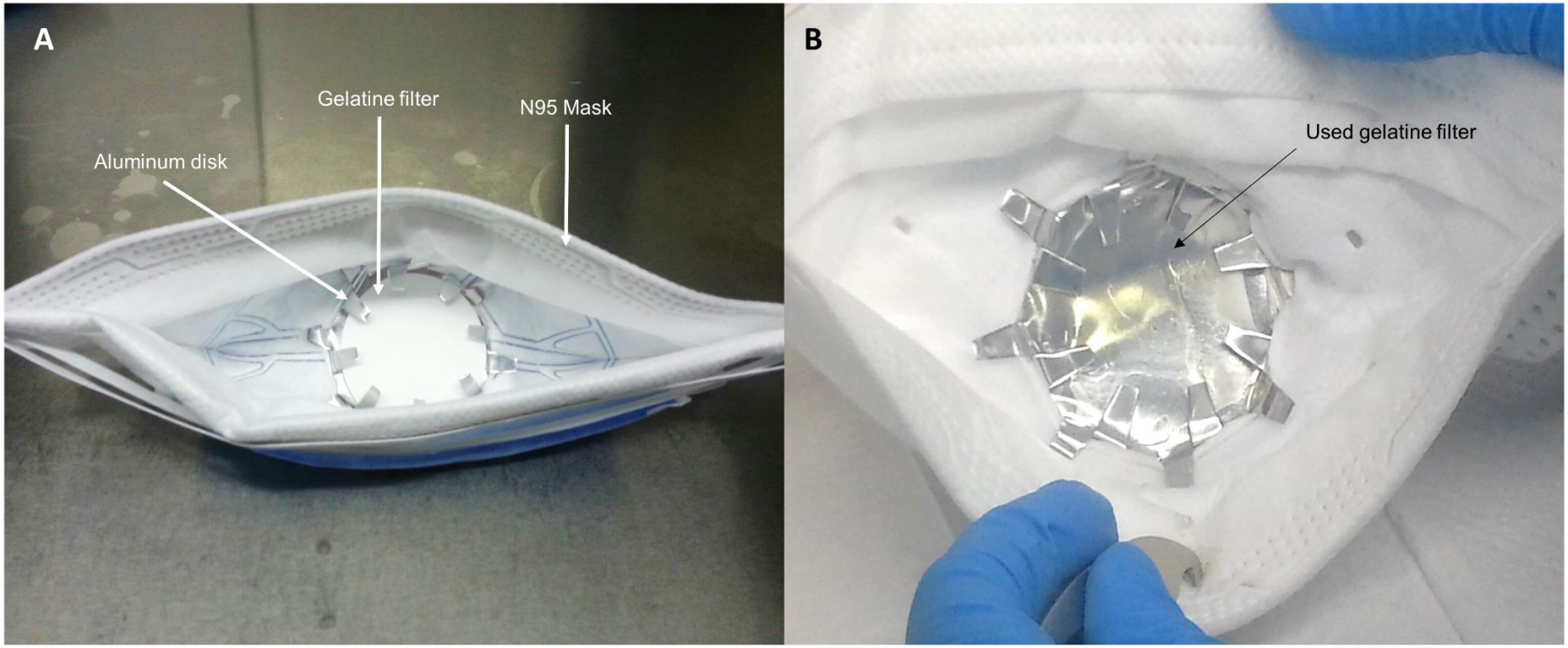
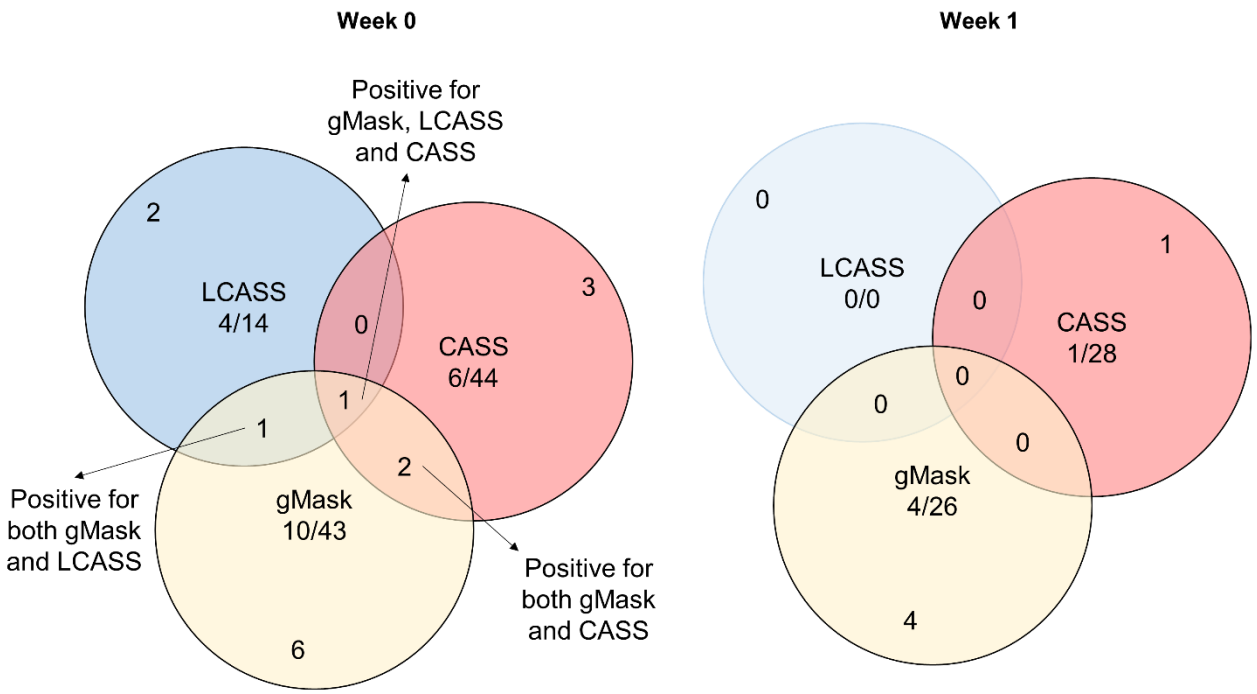


Figure 4



Tables

Table 1: Number of positive tests across time points for different aerosol sampling methods. CASS, LCASS and gMask had similar positivity at week 0. CASS and LCASS had similar rates of positivity but gMask remained positive through to week 8 of treatment. Data are n/N (%).

	Sputum		Aerosol				
Sampling time point (weeks)	**Smear microscopy**	**Culture**	**CASS**	**gMask**	**MGIT LCASS**	**7H9 LCASS**	**EPCFE LCASS**
0	23/44 (52)	31/45 (69)	6/44 (14)	10/43 (23)	4/14 (29)	0/14 (0)	0/14 (0)
1	8/23 (35)	20/27 (74)	1/28 (4)	4/26 (15)	0/12 (0)	0/12 (0)	0/12 (0)
2	6/19 (32)	16/22 (73)	0/22 (0)	2/20 (10)	0/11 (0)	0/11 (0)	0/11 (0)
4	7/14 (50)	9/14 (64)	0/14 (0)	3/14 (21)	0/9 (0)	0/9 (0)	0/9 (0)
6	0/12 (0)	6/12 (50)	0/12 (0)	2/12 (17)	0/3 (0)	0/3 (0)	0/3 (0)
8	4/11 (36)	4/11 (36)	0/10 (0)	1/10 (10)	0/3 (0)	0/3 (0)	1/3 (33)
10	2/2 (100)	2/4 (50)	0/4 (0)	0/4 (0)	0/2 (0)	0/2 (0)	0/2 (0)

At week 0, 2 patients were positive for both gMask and CASS, 1 was positive gMask and LCASS and 1 was positive for all 3 methods. At week 1 none that were gMask positive were CASS positive (see Figure 5). At week 8 the patient that was LCASS EPCFE positive was also gMask positive. Given the small denominators for time points beyond zero, p-values were not generated.

Table 2: Results of the feasibility study for which growth was seen in 3/10 (30%) of gMasks (one contaminated) and the duration study where 4/10 (40%) patients were gMask-positive. No significant difference in time to positivity (TTP) was seen with sampling duration. There were 2 patients that were gMask negative but CASS positive, indicating that gMask may miss some patients CASS detects and vice versa.

PID	Time (h)	Aerosol culture result (TTP)	Culture (TTP)	CASS result
Feasibility study				
M003	12	C (40)	N/A	N
M005	8	P (21)	N/A	N
M006	10	P(16)	N/A	P
Duration study				
M014	1	P (14)	P (4)	P
	3	P (27)		
	6	P (18)		
M016	1	P (22)	P (5)	P
	3	P (20)		
	6	P (17)		
M017	1	P (23)	P (9)	N
	3	P (3)*		
	6	P (21)		
M019	1	N	N/A+	N
	3	N		
	6	P (24) p=0.47		
gMask negative patients		n=6	n=3 P n=3 N	n=2 CASS P n=4 CASS N

* Culture was contaminated which contributed to faster TTP (however, acid fast bacilli were also visible). + Patient was ill and could not produce sputum. † Patient was ill and did not wear mask for the full 3 and 6 hours.

Table 3: Summary of patients who had at least one positive aerosol result at any time point. DS-TB patients had once-off sampling and those in DR-TB had multiple time points for both treatment arms. DR-TB patients were more likely to be positive by any methods if they were enrolled in the routine arm (20/28 vs. 8/28; p=0.003). Data are shown as n/N (%) (where N is all patients tested at that time point, not all are shown here).

		DS-TB						DR-TB													
								Routine Arm									Intervention Arm				
Week	**Sampling method**	**C364**	**C484**	**C486**	**C505**	**C478**	**C492**	**C477**	**C481**	**C509**	**C513**	**C515**	**C490**	**C504**	**C511**	**C508**	**C510**	**C488**	**C493**	**C494**	**Proportion positive**
0	**gMask**	**P**	**P**	**P**	**P**	-	-	**P**	**P**	**P**	**P**	**P**	N	N	N	N	**P**	C	N	N	10/43 (23)
	CASS	**P**	**P**		**P**	**P**	**P**	N	N	N	N	N	N	N	N	N	N	**P**	N	N	6/44 (14)
	LCASS	**P**	-	-	-	-	-	-	-	**P**	-	-	**P**	N	N	N	-	N	N	**P**	4/14 (29)
1	**gMask**	-	-	-	-	-	-	**P**	N	N	N	**P**	N	**P**	N	N	**P**†	N	N	N	4/26 (15)
	CASS	-	-	-	-	-	-	N	N	N	N	N	N	N	N	N	N	**P***	N	N	1/28 (4)
	LCASS	-	-	-	-	-	-	-	-	N	-	-	N	N	N	N	-	N	N	N	0/12 (0)
2	**gMask**	-	-	-	-	-	-	N	N	N	N	N	N	N	**P**	N	N	N	**P**	N	2/20 (10)
	CASS	-	-	-	-	-	-	N	N	N	N	N	N	N	N	N	N	C	N	N	0/22 (0)
	LCASS	-	-	-	-	-	-	-	-	N	-	-	N	N	N	N	-	N	N	N	0/11 (0)
4	**gMask**	-	-	-	-	-	-	N	**P**	N	N	N	N	**P**	N	**P**	N	N	N	N	3/14 (21)
	CASS	-	-	-	-	-	-	N	N	N	N	N	N	N	N	N	N	N	N	N	0/14 (0)
	LCASS	-	-	-	-	-	-	-	-	N	-	-	N	N	N	N	-	N	N	N	0/9 (0)
6	**gMask**	-	-	-	-	-	-	N	**P**	-	N	N	N	N	N	**P**	N	N	N	N	2/12 (17)
	CASS	-	-	-	-	-	-	N	N	-	N	N	N	N	N	N	N	N	N	N	0/12 (0)
	LCASS	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	N	N	N	0/3 (0)
8	**gMask**	-	-	-	-	-	-	N	N	-	-	-	N	N	N	N	N	N	N	**P***	1/10 (10)
	CASS	-	-	-	-	-	-	N	N	-	-	-	N	N	N	N	-	N	N	N	0/10 (0)
	LCASS	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	N	N	**P**	1/3 (0)

* These patients showed clinical signs of treatment failure and have their treatment regimens extended beyond 6 months (intervention arm). † This patient passed away at month 5 after treatment initiation (intervention arm). Grey block indicates patient positive for LCASS EPCFE indicating possible presence of DCTB in aerosol.

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Chapter 7

General conclusions and future work

We have developed methods to potentially reduce time-to-treatment by providing a way to salvage used Xpert and Ultra CE for further second-line drug susceptibility testing. This means that a full diagnosis for rifampicin, FQ and SLIDs can be obtained from a single specimen, without the need for further DNA extraction, potentially within 24 hrs. This has implications, especially in low resource settings where patients often do not return or cannot produce a second sputum of sufficient quality for further drug-susceptibility testing. Routine diagnostics laboratories can save on time and costs involved with the collection and processing of secondary sputa and the additional consequences of a patient remaining infectious and contributing to transmission[1,2]. We have further shown that potential cross-contamination of *rpoB* amplicons that may occur during the extraction process, though possible, is very unlikely, which further supports the adoption of this method. However, future work includes the development of an ease-of-use device that can clip onto the existing cartridge and extract the CE into a sealed container (Figure 1), thereby reducing the chance of needle-stick injury from the manual method and negating the chance of amplicon cross-contamination. As GeneXpert caters for a large platform of tests (e.g. HIV, gonorrhoea, methods, staphylococcus, influenza), this method can also be applied to other Xpert cartridges. This work has been funded by the C-THAN consortium.

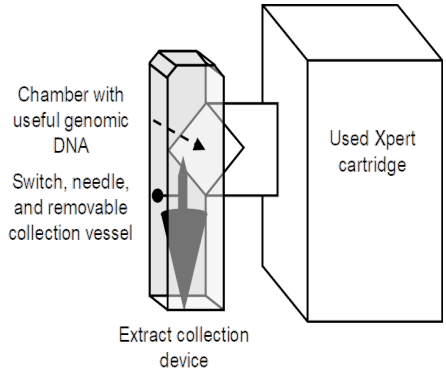

Figure 1: Example of device for salvaging cartridge extract from used cartridges.

We have also shown that CE may be useful for ultra-deep targeted next generation sequencing methods such as SMOR, and that this can give a complete antibiogram for the patient from a single specimen. However, since Ultra contains not only *rpoB* amplicons in CE but also multi-copy *IS610* and *IS1081* amplicons (amongst others), SMOR appears to yield less actionable

results on Ultra CE which warrants further optimisation. Through the addition of ThermoStop and ThermoGo to improve total reads was not feasible, there are other options to potentially reduce or remove the multiple amplicons present. One such option is to do size fractionation or separation prior to the PCR amplification step in the SMOR process which may potentially remove small interfering amplicons. This has been done previously by *Liu et al* by using lysine-functionalized silica particles to separate DNA fragments with varying sizes and may be useful for this approach[3]. The use of ultra-filtration devices, such as the Vivacon from Sartorius may also prove useful as they have been used to remove small primer fragments from larger DNA fragments (>300 bp). Another option is to use biotinylated primers and use bead pull-down methods to enrich other loci (i.e. non-*rpoB* and *IS6110.1081*) in CE prior to SMOR amplification.

Further investigation into the clinical relevance of heteroresistance detected by SMOR on CE, and missed by conventional DST methods, must also be done to determine if this heteroresistance is also present in subsequent specimens in the diagnostic pipeline (e.g. sub-culturing steps during DST) and whether it affects the clinical outcome of these patients.

We also aimed to improve culturablity of DCTB in sputa of patients undergoing MDR-TB treatment (routine and intervention arms). This has been shown in patients undergoing DS-TB treatment but never in patients undergoing MDR-TB treatment, including the regimen containing bedaquiline and linezolid[7]. While there was no significance difference seen at specific time points, in the total bacilli detected between MPN assays including EPCFE and standard 7H9 cultures, there were more total bacilli detected (including DCTB) using EPCFE MPN assays over all, thus suggesting that a sub-population of DCTB not detected by conventional methods is present throughout treatment. The significance per time point may be improved with larger study numbers. Importantly, including EPCFE in the MPN assay improved detection of patients with only DCTB present through treatment that would otherwise

have been missed by standard methods. However, this did not inform on potential patient outcomes.

Furthermore, Improving Xpert for treatment monitoring by pre-treating sputa with a photoactive to reduce signals from non-viable bacteria proved not to be feasible. This is possibly due to DNA present from other bacteria and viruses in the microbiota of the sputum that sequesters the dye before it can reach the TB bacteria, which are present in smaller numbers. Furthermore, the cell wall of TB is known to be much tougher to penetrate and may also be a barrier for the dye. Previous studies have looked at the effect of such dyes, such as PMA, to quantify viable cells both *in vitro* and in clinical samples collected from patients undergoing DS-TB treatment, and while it did not seem to influence results in presence of most drugs, it had effects when INH and EMB was used[4]. PMA also increased the C_T values through treatment, however, as in our study, the changes seen were not as large as described previously by Miotto *et al*[4,5] though it was significant. Their results may be due to the treatment of the sputa with Sputasol which Nikolayevskyy *et al* have shown inactivates PMA[6]. This is the reason we treated out samples with N-acetyl-cysteine (without NaOH), however, our results still indicate that, while there is a change in $\Delta C_{Tmin,}$ it is not significant and does not improve Xpert for treatment monitoring purposes. This may be attributed to the fact that many patients enrolled in the study had already undergone 2 weeks of treatment and thus skewed the results.

Finally we have shown two new methods of aerosol sampling, using the only validated means of aerosol sampling in humans to date, the CASS, as reference standard[8-10]. Previous studies by Williams *et al* looked at using face masks as sampling tools for aerosols and their findings support the use of this non-invasive, low cost way of sampling for aerosols produced by patients with pulmonary TB. Their studies looked at a smaller sample size and used either Xpert or PCR to quantify the aerosols[11,12]. While that is indeed a much faster method of diagnosis, we opted to culture the gelatine masks to determine if culturable bacilli were being produced, as PCR methods do not differentiate between DNA from viable and non-viable bacilli. Furthermore,

while they sampled mainly drug-susceptible patients, we also wanted to sample patients with MDR-TB and sample them longitudinally to determine how treatment may affect aerosol production. We likewise found that the gMasks were a viable way of sampling for aerosols and that indeed, they detected culturable bacteria in aerosol further into treatment than the CASS (or LCASS), which relies of 5 min forced coughing. It was also found by Kennedy *et al* that these masks could be used for testing patients with COPD, thus there is utility outside the field of tuberculosis research, however the gelatine filter interfered with the detection of antimicrobial resistance (AMR) genes[13]. Further investigations using this mask can include the use of cellulose acetate membranes as they have been shown to have better bacterial recovery than other filters, and may reduce the interference seen in AMR detection[14]. Shaikh *et al* also showed the feasibility of RNA isolation from face masks that could be used to quantify viable bacilli from aerosols, which is promising[14].

The LCASS, which was by designed by us and based on the modified CASS has, to our knowledge, not been described to date. This method has potential to be used with various culturing media and methods such as including EPCFE to detect growth of DCTB in aerosol and may inform on patient infectiousness through treatment. While CASS and LCASS were done on the same patients, they were not done on the same day and so variation in aerosol production may occur. Furthermore, many of the aerosol sampling were done on patients already on treatment for up to 2 weeks. Therefore, in future studies, these methods should be sampled on treatment naïve patients and potentially at the same time to ascertain the true worth of these methods. Furthermore, this study only looked at 5 min forced cough. Patterson *et al* have shown that *Mtb* aerosols are produced even during tidal breathing[15], and likewise Williams *et al* detected *Mtb* in mask samples in hospitalised patients, often thought too sick to produce aerosols, when no coughing occurred[11]. It would therefore be pertinent to test these sampling methods on not only forced cough, but also tidal breathing or talking for extended periods.

In conclusion, this study examines at various ways in which DR-TB transmission can ultimately be reduced throughout the care cascade. We have identified ways to reduce the need for a second sputum collection and effectively reduce time-to-treatment of patients with DR-TB (rifampicin and second-line drug susceptibly testing), amongst other downstream uses. We have also shown that DCTB are present throughout treatment and may be missed by conventional tests. Finally, we have developed and tested novel methods of sampling aerosols from patients. These results can inform routine diagnostic pathways and management of patients on treatment for DR-TB.

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Appendix I

Xpert MTB/RIF results in Patients with previous Tuberculosis: Can we distinguish true from false positive results?

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MAJOR ARTICLE

Xpert MTB/RIF Results in Patients With Previous Tuberculosis: Can We Distinguish True From False Positive Results?

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Background. Patients with previous tuberculosis may have residual DNA in sputum that confounds nucleic acid amplification tests such as Xpert MTB/RIF. Little is known about the frequency of Xpert-positive, culture-negative ("false positive") results in retreatment patients, whether these are distinguishable from true positives, and whether Xpert's automated filter-based wash step reduces false positivity by removing residual DNA associated with nonintact cells.

Methods. Pretreatment patients (n = 2889) with symptoms of tuberculosis from Cape Town, South Africa, underwent a sputum-based liquid culture and Xpert. We also compared Xpert results from dilutions of intact or heat-lysed and mechanically lysed bacilli.

Results. Retreatment cases were more likely to be Xpert false-positive (45/321 Xpert-positive retreatment cases were false-positive) than new cases (40/461) (14% [95% confidence interval {CI}, 10%-18%] vs 8% [95% CI, 6%–12%]; $P = .018$). Fewer years since treatment completion (adjusted odds ratio [aOR], 0.85 [95% CI, .73–.99]), less mycobacterial DNA (aOR, 1.14 [95% CI, 1.03–1.27] per cycle threshold [C_T]), and a chest radiograph not suggestive of active tuberculosis (aOR, 0.22 [95% CI, .06–.82]) were associated with false positivity. C_T had suboptimal accuracy for false positivity: 46% of Xpert-positives with $C_T > 30$ would be false positive, although 70% of false positives would be missed. C_T's predictive ability (area under the curve, 0.83 [95% CI, .76–.90]) was not improved by additional variables. Xpert detected nonviable, nonintact bacilli without a change in C_T vs controls.

Conclusions. One in 7 Xpert-positive retreatment patients were culture negative and potentially false positive. False positivity was associated with recent previous tuberculosis, high C_T, and a chest radiograph not suggestive of active tuberculosis. Clinicians may consider awaiting confirmatory testing in retreatment patients with $C_T > 30$; however, most false positives fall below this cut-point. Xpert can detect DNA from nonviable, nonintact bacilli.

Keywords. tuberculosis; diagnosis; Xpert; false positivity.

Xpert MTB/RIF (Xpert; Cepheid) is an automated nucleic acid amplification test (NAAT) for *Mycobacterium tuberculosis* and rifampicin resistance [1–3], endorsed by the World Health Organization and the US Food and Drug Administration [4, 5]. Xpert is increasingly deployed in many countries as the initial diagnostic test for tuberculosis [6].

Xpert is used routinely in patients who have previously had tuberculosis [6, 7]. This is despite evidence that approximately 30% of patients who are microbiologically cured after 6 months of treatment are Xpert positive [8], a proven correlation between retreatment status and diminished specificity [9–11], and several case reports detailing false-positive (FP) Xpert results in retreatment cases [12–14]. Detectable mycobacterial DNA, which can be extracellular or associated with nonintact cells (and hence is not culturable), is a possible cause of this false positivity, which may trigger unwarranted treatment and unnecessarily expose patients to toxic drugs, delay establishing the correct underlying diagnosis and its appropriate treatment, and escalate healthcare costs. Although the manufacturer recommends that Xpert always be used in conjunction with culture [15], culture capacity is not mandatory for Xpert's use in the field [7] and, even in high-burden countries such as South Africa that do have culture capacity, most Xpert-positive patients do not receive culture, as per the national algorithm [16].

More than 700 000 patients with a history of tuberculosis were diagnosed in 2013 [17]; however, there are limited data about the frequency of Xpert false positivity in retreatment patients [18] and what factors, if any, may guide clinical practice [10]. We therefore examined the relationship between Xpert results (including *M. tuberculosis* complex–specific quantitative information), routinely collected clinical information, and

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culture results in a large cohort of patients evaluated for tuberculosis in the high-burden, high–human immunodeficiency virus (HIV) setting of Cape Town, South Africa. To interrogate claims that Xpert does not detect free DNA because of a preamplification wash step [19–22], which would potentially reduce the risk of an FP result in retreatment cases, we performed a laboratory-based substudy to ascertain whether Xpert can detect DNA from lysed nonviable cells.

METHODS

Patient Recruitment

We analyzed data from 3166 patients who had symptoms suggestive of tuberculosis. Patients were recruited from primary care clinics or hospitals in Cape Town, South Africa, as part of studies that evaluated the utility of Xpert. Patients included in the final analysis were Xpert positive and had cycle threshold (C_T) data, were either culture positive or negative, had a known previous tuberculosis status, had not been on treatment for >48 hours, and had not taken antituberculosis treatment 60 days prior to testing. This study was approved by the University of Cape Town Faculty of Health Sciences Ethics Committee.

Diagnostic Tests

Two paired sputum specimens were collected at recruitment; 1 was randomly selected for an Xpert test, and the other was used for a BACTEC MGIT 960 liquid culture (Becton, Dickinson, and Co). If patients were unable to expectorate sputum, sputum induction with hypertonic saline was performed. Tuberculosis morbidity score [23] data and chest radiograph (CXR) data were collected in a subset of patients, as determined by the parent protocol.

Xpert MTB/RIF Cell Lysis Experiment

To assess whether Xpert detected nonviable cells, 1 mL of *M. tuberculosis* H37Rv in phosphate-buffered saline and 0.25% Tween 80 (10 000, 1000, 500, and 0 colony-forming units [CFU] mL^{-1}) was added to Xpert sample buffer (2:1 ratio) and, after 15 minutes of incubation with intermittent shaking, 2 mL was added to the Xpert cartridge (direct Xpert). In parallel, a 1.5-mL aliquot of each concentration underwent heat treatment (80°C, 1 hour), followed by mechanical disruption using Lysing Matrix B tubes (0.1 mm zirconium beads; MP Biochemicals) and a Fast Prep-24 machine (MP Biochemicals) (6.5 meters per second for three 30-second intervals with 1 minute resting on ice between intervals). After bead-beating, the lysate was allowed to settle for 2 minutes and 1 mL of supernatant was used for Xpert (lysed Xpert). Ten 10-µL aliquots of each dilution (direct and lysed) were plated on Middlebrook 7H10 agar supplement with oleic acid albumin dextrose complex and incubated for 6 weeks at 37°C to check for viability. This experiment was performed in triplicate.

Statistical Analysis

Xpert-positive, culture-positive patients were defined as true positive (TP) and Xpert-positive, culture-negative patients were defined as false positive (FP). The χ^2 test was used for comparisons between proportions. The Mann–Whitney test was used to compare differences in nonparametric continuous data. Multivariable logistic regression was performed to adjust for potential confounding. A backward elimination strategy using the likelihood ratio test was used to finalize each model. Analyses were performed using GraphPad Prism version 6.0 (GraphPad Software) and Stata version 13 (StataCorp) software. All statistical tests are 2-sided at $\alpha = .05$.

RESULTS

Of the 3166 patients, we excluded 263 (8%) patients (73 had did not have a positive- or negative-culture result, 86 were on treatment >48 hours, 104 Xpert-positive patients were missing C_T data, and 14 were missing data on their previous tuberculosis history). Of the remaining 2889 patients with a known culture status, 837 (29%) were culture positive and 782 (27%) were Xpert positive. A total of 1220 (42%) patients were retreatment cases. A summary of the demographic and clinical characteristics of the cohort is shown according to previous tuberculosis status in Table 1. Retreatment patients were more likely to be older and HIV-infected. Differences in Xpert C_T and years since completion of previous antituberculosis treatment in new and retreatment patients are shown in Figure 1.

Xpert MTB/RIF False Positivity in Patients With Newly Diagnosed Tuberculosis

Xpert had a sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of 83% (95% confidence interval [CI], 80%–86%), 97% (95% CI, 95%–98%), 91% (95% CI, 88%–94%), and 93% (95% CI, 91%–94%), respectively, in new patients. Forty of 461 (9%) Xpert-positive results were FP.

Table 1. Demographic and Clinical Characteristics of Patients Included in the Analysis Who Had Symptoms Suggestive of Tuberculosis

Characteristic	No Previous TB (n = 1669)	Previous TB (n = 1220)	*P* Value
Demographic characteristics			
Female sex, No. (%)	773/1617 (48)	548/1181 (46)	.463
Age, y, median (IQR)	36 (28–46)	39 (32–49)	<.001
Clinical characteristics			
HIV-infected, No. (%)	695/1617 (42)	623/1198 (53)	<.001
TB morbidity score, median (IQR)	5 (3–6)	5 (3–6)	.654
Test characteristics			
Culture-positive, No. (%)	506/1669 (30)	331/1220 (27)	.062
Time-to-positivity, d, median (IQR)	12 (8–17)	13 (8–17)	.352
Xpert MTB/RIF-positive, No. (%)	461/1669 (28)	321/1220 (26)	.434
C_T, median (IQR)	21.20 (17.92–26.64)	22.00 (17.23–27.06)	.309
CXR compatible with active TB, No. (%)	402/928 (43)	329/708 (46)	.204

Abbreviations: C_T, cycle threshold; CXR, chest radiograph; HIV, human immunodeficiency virus; IQR, interquartile range; TB, tuberculosis.

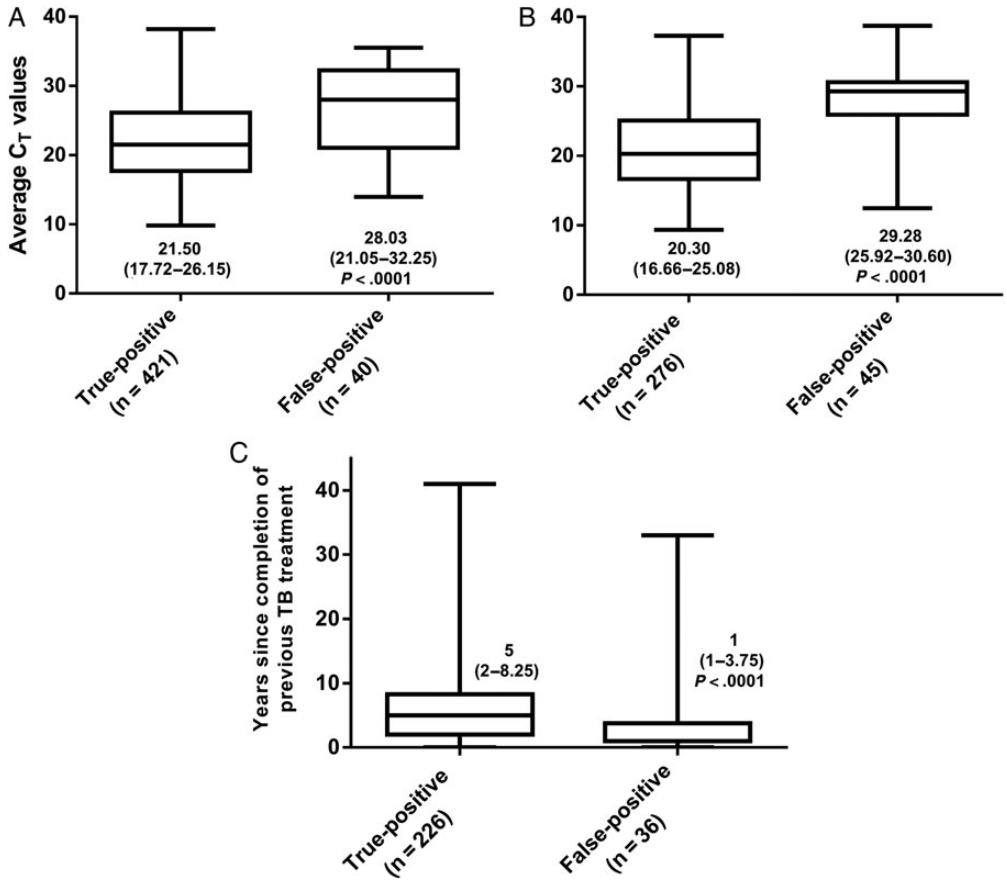

Figure 1. Box-and-whisker plot comparison of Xpert MTB/RIF quantitative information (cycle threshold [C_T] values) in true-positive (Xpert-positive, culture-positive) and false-positive (Xpert-positive, culture-negative) specimens for new (*A*) or retreatment (*B*) patients, and a comparison of the years since the completion of previous tuberculosis (TB) treatment in Xpert-positive retreatment patients according to culture status (*C*). Median values with interquartile ranges in parentheses are shown.

Correlates of Xpert MTB/RIF False Positivity

New tuberculosis patients with an FP Xpert were, compared to those who were TP, more likely to have higher median Xpert C_T (28.03 [interquartile range {IQR}, 21.20–32.23] vs 21.50 [IQR, 17.72–26.10]; $P < .001$) and more likely to be female (25/40 [63%] of FP cases were women vs 187/414 [45%] of TP cases; odds ratio [OR], 2.02 [95% CI, 1.04–3.95]). In a multivariate logistic regression analysis (Table 2), each unit increase in C_T was associated with a 14% increase in the relative risk (adjusted OR [aOR], 1.14 [95% CI, 1.08–1.21]) of Xpert false positivity, presuming the other variables held constant. Morbidity in patients with tuberculosis symptom score data (n = 168) was similar in those with an FP or TP Xpert result (median, 4 [IQR, 2–6] vs 4 [IQR, 4–5]; $P = .389$).

Where CXR data (n = 193) were available, FP patients were less likely to have a CXR compatible with active tuberculosis than TP patients (9/19 [47%] vs 144/174 [83%]; $P = .001$) and, when included in a multivariable logistic regression model with previous tuberculosis and C_T, a CXR compatible with active tuberculosis was associated with a 79% reduction in the relative odds of Xpert false positivity (aOR, 0.21 [95% CI, .08–.57]; Supplementary Table 2).

Receiver Operating Characteristic Curve Analyses

The area under the curve (AUC) for C_T (0.70 [95% CI, .61–.80]) did not increase when CXR (0.78 [95% CI, .71–.86]) was included (Figure 2) (receiver operating characteristic [ROC] curves for C_T only, and not those who also had CXR data, are shown in Supplementary Figure 1). At a rule-out cut-point ($C_T > 14.22$) for Xpert false positivity (selected based on 95% sensitivity; ie, 95% of the 40 FP Xperts fell above this cut-point), C_T alone had a specificity, NPV and negative likelihood ratio (LR) of 6%, 93%, and 0.78, respectively (Table 3). At a rule-in cut-point (selected based on 95% specificity) of >32.19, C_T had a sensitivity, PPV, and positive LR of 3%, 33%, and 5.5, respectively. At a cut-point ($C_T > 27.08$) corresponding to Youden index, C_T had a sensitivity, specificity, NPV, PPV, positive LR, and negative LR of 55%, 80%, 21%, 95%, 2.79, and 0.56, respectively.

Xpert MTB/RIF False Positivity in Retreatment Tuberculosis Patients

Xpert had a sensitivity, specificity, PPV, and NPV of 84% (95% CI, 79%–87%), 94% (95% CI, 93%–96%), 85% (95% CI, 82%–

Table 2. Factors Associated With Xpert False Positivity in New and Retreatment Cases

	Univariate Analysis				Multivariate Logistic Regression	
New TB Patients (n = 461)	True-Positive Xpert (n = 421)	False-Positive Xpert (n = 40)	OR (95% CI)	*P* Value	Adjusted OR (95% CI)	*P* Value
Demographic variables						
Age, y, median (IQR)	33 (27–43)	32 (25–44)	1.00 (.97–1.02)	.780	. . .	. . .
Female, No. (%)	187/414 (45)	25/40 (63)	2.02 (1.04–3.95)	.039	. . .	. . .
Smoker, No. (%)	125/347 (36)	13/30 (43)	1.36 (.64–2.89)	.427	. . .	. . .
Clinical variables						
HIV-infected, No. (%)	162/404 (58)	21/39 (50)	1.74 (.90–3.37)	.099	. . .	. . .
Xpert information						
TB-specific C_T values, median (IQR)	21.50 (17.72–26.10)	28.03 (21.20–32.23)	1.14 (1.07–1.21)	<.001	1.14 (1.08–1.21)	<.001
Retreatment Patients (n = 321)	True-Positive Xpert (n = 276)	False-Positive Xpert (n = 45)	OR (95% CI)	*P* Value	Adjusted OR (95% CI)	*P* Value
Demographic variables						
Age, y, median (IQR)	37 (30–45)	41 (21–48)	1.03 (1–1.07)	.030	. . .	. . .
Female, No. (%)	108/274 (39)	15/42 (36)	1.17 (.60–2.30)	.647	. . .	. . .
Smoker, No. (%)	100/229 (44)	18/32 (56)	1.66 (.79–3.50)	.184	. . .	. . .
Clinical variables						
HIV-infected, No. (%)	122/273 (45)	20/43 (47)	1.08 (.57–2.05)	.823	. . .	. . .
Previous TB treatment not completed, No. (%)	55/239 (23)	26/39 (26)	1.15 (.53–2.51)	.719	. . .	. . .
Years since previous TB treatment stopped or completed, median (IQR)	2 (0–5)	1 (0–1)	0.92 (.85–.99)	.033	0.91 (.84–.99)	.048
Xpert information						
TB-specific C_T values, median (IQR)	20.30 (16.71–25.05)	29.28 (26.18–30.60)	1.27 (1.18–1.37)	<.001	1.25 (1.15–1.35)	<.001

Versions of this table for all patients (Supplementary Table 1) or restricted to the subset of patients with chest radiographic data (Supplementary Table 2) are provided in the Supplementary Data.
Abbreviations: CI, confidence interval; C_T, cycle threshold; HIV, human immunodeficiency virus; IQR, interquartile range; OR, odds ratio; TB, tuberculosis.

90%), and 94% (95% CI, 92%–95%) in retreatment patients. Forty-five of 321 (14%) Xpert-positive results were false positive ($P = .018$ compared to new cases).

Correlates of Xpert MTB/RIF False Positivity

Although retreatment patients with an FP Xpert were older than those with a TP Xpert in a univariate analysis (Median [IQR], 41 {21–48} vs 37 {30–45} years; $P = .030$), after multivariable adjustments were performed, only C_T (aOR, 1.25 [95% CI, 1.15–1.35]; $P < .001$) and the number of years since stopping treatment for the previous episode of tuberculosis (aOR, 0.91 [95% CI, .84–.99]; $P = .048$) were independent predictors of Xpert FP (Table 2). There was no correlation between C_T and years since stopping treatment for previous tuberculosis ($P = .427$; Supplementary Figure 2). When radiographic data were available, a CXR compatible with active tuberculosis was also an independent predictor of Xpert false positivity (aOR, 0.22 [95% CI, .06–.82]; Supplementary Table 2).

Receiver Operating Characteristic Curve Analyses

ROC curve AUCs of 0.83 (95% CI, .76–.90), 0.83 (95% CI, .75–.91), 0.78 (95% CI, .79–.86), and 0.84 (95% CI, .72–.95) were obtained for C_T alone, a model incorporating C_T and the number of years since stopping treatment, a model incorporating C_T and CXR, and a model incorporating all 3 variables, respectively (Figure 2). C_T had, at a cut-point for ruling out Xpert false positivity (selected based on 95% sensitivity; ie, 95% of the 45 FP Xperts in retreatment patients fell above this cut-point), a specificity, NPV, and negative LR of 36%, 98%, and 0.12, respectively (cut-point >18.28), whereas at a rule-in cut-point (selected based on 95% specificity), it had a sensitivity, PPV, and positive LR of 27%, 46%, and 5.26, respectively (cut-point >30.56) (Table 3). At a cut-point (>28.36) corresponding to Youden index, C_T had a sensitivity, specificity, NPV, PPV, positive LR, and negative LR of 64%, 90%, 52%, 94%, 6.59, and 0.39, respectively.

Detection of DNA From Nonviable Bacilli

Each dilution of bacilli (10 000, 1000, and 500 CFU mL^{-1}) was detected as positive when Xpert was done directly or on lysate. Similar C_Ts (SEM) were obtained (direct vs lysed): 16.58 (0.70) vs 16.98 (0.98; $P = .826$), 19.03 (0.53) vs 21.04 (0.88; $P = .266$), and 21.15 (0.37) vs 21.62 (0.86; $P = .730$) for the 10 000, 1000, and 500 CFU mL^{-1} dilutions, respectively (Figure 3). The 0 CFU mL^{-1} dilutions were undetected. After 6 weeks of incubation, each aliquot used for direct Xpert grew the expected number of CFUs, whereas no growth was observed from the aliquots of heat inactivated, bead-beaten bacilli.

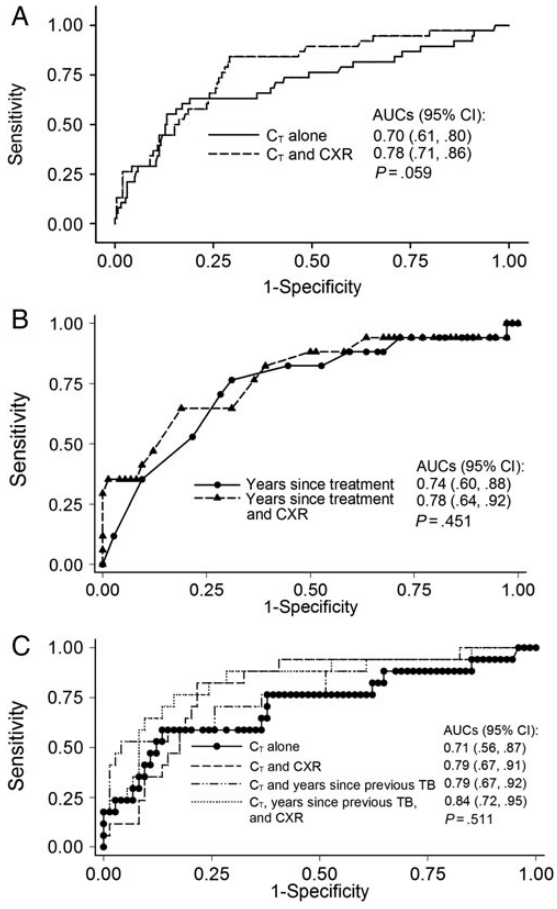

Figure 2. Receiver operating characteristic curves of Xpert MTB/RIF cycle threshold values and clinical information for the prediction of Xpert MTB/RIF false positivity in new (*A*) or retreatment (*B* and *C*) patients. Abbreviations: AUC, area under the curve; CI, confidence interval; C_T, cycle threshold; CXR, chest radiograph; TB, tuberculosis.

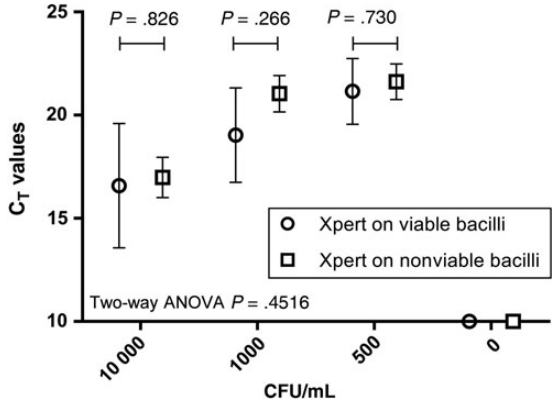

Figure 3. Comparison of Xpert MTB/RIF cycle threshold values (mean ± SEM) from a dilution series of bacilli, showing similar C_T when Xpert MTB/RIF was performed on intact bacilli or nonviable, heat- and mechanically-lysed bacilli. Three experimental replicates were performed. Abbreviations: ANOVA, analysis of variance; CFU, colony-forming units; C_T, cycle threshold; SEM, standard error of the mean.

DISCUSSION

Our key findings are as follows: (1) patients with an FP Xpert are more likely to have previous tuberculosis (and to have had this more recently), low mycobacterial DNA load (measured by C_T), and a CXR not compatible with active tuberculosis; (2) about 1 in 7 Xpert-positive results in retreatment patients will be FP; (3) C_T predicts false positivity, but has suboptimal discriminatory power (a specificity of 10% at a rule-out cut-point [95% sensitivity], and a sensitivity of 20% at a rule-in cut-point [95% specificity]) that is not enhanced by the incorporation of additional variables; (4) using a cut-point of $C_T > 30$ in retreatment patients, 7 of 10 FP cases will be missed; however, about half of the patients falling above this cut-point will be FP; and (5) Xpert detects DNA from nonviable cells that are not intact, thereby suggesting that free DNA—and not just DNA from intact cells—is detected by Xpert.

Early evaluations of Xpert [25] contributed the majority of data to meta-analyses of test accuracy [26, 27]; however these studies excluded patients who were culture-negative and treated based on symptoms (including many Xpert-positive patients), despite the known poor specificity of empirical treatment [28, 29]. This led to calls that Xpert's specificity might be overestimated, especially in retreatment cases [12, 17]. A reanalysis of the pooled data found that, when these early evaluations were excluded, no significant change in specificity occurred; however,

Table 3. Accuracy of Cycle Threshold Values for Predicting Xpert MTB/RIF False Positivity in New and Retreatment Patients

Test Use	Suggested C_T Cut-point	Sensitivity, % (95% CI)	Specificity, % (95% CI)	PPV, % (95% CI)	NPV, % (95% CI)	Positive LR (95% CI)	Negative LR (95% CI)
New patients (n = 461)							
Rule-in	>32.19	3 (2–4)	95 (92–97)	34 (19–.54)	93 (90–95)	5.51 (2.87–10.60)	0.76 (.63–.92)
Rule-out	>14.22	95 (82–99)	6 (4–9)	9 (6–12)	93 (76–99)	1.02 (.94–1.09)	0.78 (.19–3.22)
Youden index[a]	>27.08	55 (39–70)	80 (76–84)	21 (14–30)	95 (92–97)	2.79 (1.99–3.92)	0.56 (.40–.79)
Retreatment patients (n = 321)							
Rule-in	>30.56	27 (15–42)	95 (91–97)	46 (27–66)	89 (85–92)	5.26 (2.60–10.63)	0.77 (.65–.92)
Rule-out	>26.80	95 (84–99)	36 (30–42)	20 (15–26)	98 (92–100)	1.39 (1.33–1.66)	0.12 (.03–.49)
Youden index[a]	>28.36	64 (49–78)	90 (86–93)	52 (38–65)	94 (90–96)	6.59 (4.33–10.01)	0.39 (.27–.58)

Abbreviations: CI, confidence interval; C_T, cycle threshold; LR, likelihood ratio; NPV, negative predictive value; PPV, positive predictive value.

[a] Defined as the best compromise between sensitivity and specificity assuming equal weighting [24].

when patients' history of previous tuberculosis was included as a covariate, a trend between an increased prevalence of retreatment cases and diminished specificity existed [9].

The specificity of Xpert in retreatment cases in our study was 95% (95% CI, 93%–96%), indicating that 1 in 20 culture-negative patients will be FP by Xpert. This is less than the specificity reported in meta-analyses that included (99% [95% CI, 98%–99%]) [9] or excluded (98% [95% CI, 97%–99%]) data from the initial Xpert validation studies [25]. Our specificity is also less than that reported previously in retreatment cases in South Africa (99% [95% CI, 98%–100%]) [11], but higher than that seen among retreatment cases in Harare (87% [95% CI, 75%–94%]) [10]. As suggested by others [10, 11], our study indicates that about 1 in 7 Xpert-positive retreatment patients will be FP. In settings such as Cape Town, South Africa, where approximately 1 in 4 tuberculosis notifications are retreatment cases (approximately 7500 per annum) [30], this represents a potentially large public health problem.

We found C_T to differentiate poorly between TP or FP Xpert results. For example, at an optimized rule-out cut-point (C_T > 26.80; 95% sensitivity) in retreatment patients, only a third of true-positive patients would be correctly classified, and only 1 in 5 FP Xpert results would be correctly classified. Conversely, 70% of FP cases would be missed at an optimized rule-out cut-point (C_T > 30.56; 95% specificity), and less than half of the Xpert-positive results with C_T above this cut-point would be correctly classified as FP. Although suboptimal for use in routine clinical practice, this result suggests that clinicians should be cautious in interpreting Xpert-positive results in retreatment patients with $C_T > 30$, and that they may wish to await the results of confirmatory culture-based testing before starting treatment. This study, as well as others that have demonstrated C_T to be a useful proxy of bacterial load [31–33] and infectiousness [34–36], suggests that laboratories should consider routinely reporting these values.

Our study is the first to describe an inverse association between Xpert FP results and the time since previous treatment was stopped, and the utility of CXR in discriminating Xpert TP from FP patients. Although these tools reduced the odds of an FP result, they did not, unfortunately, improve upon the relatively poor discriminatory ability of C_T alone. This is because several TP patients had recently been treated for active tuberculosis (which is reflective of our high transmission setting), or had a CXR not suggestive of active tuberculosis.

As Xpert does not detect DNA from nontuberculous mycobacteria [37], almost all positive results likely reflect the true detection of *M. tuberculosis* complex DNA [32]; however, this does not always correspond to the presence of active disease caused by viable, intact bacilli. Our research shows that the on-board sample processing system of Xpert is unable to remove genomic DNA from nonintact, nonviable cells, which may be present in retreatment cases. This is likely the mechanism by which Xpert FP occurs, and suggests that Xpert's automated mechanism to isolate intact bacilli prior to DNA extraction requires optimization if false positivity due to the detection of extracellular DNA or DNA in nonintact, nonviable cells is to be minimized. Notably, a study observed Xpert to effectively remove large numbers of amplicons in spiked sputum, preventing detection [37]; however, unlike our study, this earlier work used free DNA of low molecular weight.

Our study has limitations. Other causes of Xpert false positivity include variations in specimen quality and bacterial load in the different samples used for Xpert and culture, and the overlapping stochastic limits of detection of these 2 tests, which can cause false-negative reference standard results (and hence false-positive Xpert results, which may be minimized by repeated cultures). Culture itself is an imperfect reference standard with incomplete sensitivity, although it is used widely in both clinical practice and research. Several factors may underpin this incomplete sensitivity including sampling error, differential immune reactivity in retreatment cases, and technical reasons, among others. Furthermore, we lacked long-term systematic clinical outcome data to incorporate into a reference standard; however, this lacks specificity as empiric overtreatment is frequent in high-burden settings [28, 38, 39], patients without tuberculosis can still improve when on antituberculosis treatment, and patients with tuberculosis and a concomitant infection (eg, Pneumocystis) can still fail to improve. Laboratory error and sample cross-contamination are, as always, potential sources of error; however, Xpert is a closed system that generates few aerosols [40], and we performed Xpert in a quality-assured laboratory separate to that used for culture. Finally, it should also be noted that these findings, which are from a high-burden setting with a high intensity of transmission and where retreatment tuberculosis is relatively common, should undergo further validation, especially in different settings.

In summary, patients with a history of tuberculosis, more recent previous tuberculosis, and a CXR incompatible with active tuberculosis are at a higher risk of Xpert false positivity; however, these do not add discriminatory power over and above C_T alone. Although most FP cases would be missed, clinicians should treat $C_T > 30$ in retreatment cases with caution. Further investigation is needed to discriminate NAAT FP patients from TP patients, including research into technologies that exclude DNA from nonintact cells (such as propidium monoazide or ethidium monoazide staining [41, 42]) or detect messenger RNA in live bacilli [43]. This is important as next-generation NAATs, such as Xpert Ultra, will purportedly have a sensitivity approaching that of culture [44], and hence be more likely to detect low quantities of residual tuberculosis DNA and have poor specificity in patients who have previously had tuberculosis.

Supplementary Data

Supplementary materials are available at http://cid.oxfordjournals.org. Consisting of data provided by the author to benefit the reader, the posted

materials are not copyedited and are the sole responsibility of the author, so questions or comments should be addressed to the author.

Notes

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Author contributions. Conception and design: G. T. and K. D. Collection of data: all authors. Analysis and first draft: G. T. and K. D. Interpretation and important intellectual input: all authors.

Potential conflicts of interest. All authors: No reported conflicts. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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Appendix II

False positive Xpert MTB/RIF results in re-tested patients with previous tuberculosis: frequency, profile, and prospective clinical outcomes

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False-Positive Xpert MTB/RIF Results in Retested Patients with Previous Tuberculosis: Frequency, Profile, and Prospective Clinical Outcomes

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ABSTRACT Globally, Xpert MTB/RIF (Xpert) is the most widely used PCR test for the diagnosis of tuberculosis (TB). Positive results in previously treated patients, which are due to old DNA or active disease, are a diagnostic dilemma. We prospectively retested sputum from 238 patients, irrespective of current symptoms, who were previously diagnosed to be Xpert positive and treated successfully. Patients who retested as Xpert positive and culture negative were exhaustively investigated (repeat culture, chest radiography, bronchoscopy with bronchoalveolar lavage, long-term clinical follow-up). We evaluated whether the duration since previous treatment completion, mycobacterial burden (the Xpert cycle threshold [C_T] value), and reclassification of Xpert-positive results with a very low semiquantitation level to Xpert-negative results reduced the rate of false positivity. A total of 229/238 (96%) of patients were culture negative. Sixteen of 229 (7%) were Xpert positive a median of 11 months (interquartile range, 5 to 19 months) after treatment completion. The specificity was 93% (95% confidence interval [CI], 89 to 96%). Nine of 15 (40%) Xpert-positive, culture-negative patients reverted to Xpert negative after 2 to 3 months (1 patient declined further participation). Patients with false-positive Xpert results had a lower mycobacterial burden than patients with true-positive Xpert results (C_T, 28.7 [95% CI, 27.2 to 30.4] versus 17.6 [95% CI, 16.9 to 18.2]; $P < 0.001$), an increased likelihood of a chest radiograph not compatible with active TB (5/15 patients versus 0/5 patients; $P = 0.026$), and less-viscous sputum (15/16 patients versus 2/5 patients whose sputum was graded as mucoid or less; $P = 0.038$). All patients who initially retested as Xpert positive and culture negative ("Xpert false positive") were clinically well without treatment after follow-up. The duration since the previous treatment poorly predicted false-positive results (a duration of ≤2 years identified only 66% of patients with false-positive results). Reclassifying Xpert-positive results with a very low semiquantitation level to Xpert negative improved the specificity (+3% [95% CI, +2 to +5%]) but reduced the sensitivity (−10% [95% CI, −4 to −15%]). Patients with previous TB retested with Xpert can have false-positive results and thus not require treatment. These data inform clinical practice by highlighting the challenges in interpreting Xpert-positive results, underscore the need for culture, and have implications for next-generation ultrasensitive tests.

KEYWORD *Mycobacterium tuberculosis*

Xpert MTB/RIF (Xpert) is a widely used DNA-based PCR test for the diagnosis of active tuberculosis (TB) (1). Mycobacterial DNA and Xpert positivity can persist in the absence of culturable bacilli for years after treatment (2–4). However, the signifi-

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cance of Xpert-positive, culture-negative results (Xpert false-positive results) remains uncertain because culture is an imperfect "gold standard" susceptible to sampling error and technical variation (5, 6). Understanding these Xpert false-positive results is critical given the large global burden of symptomatic patients who present for investigation for active TB but have previously been treated for active TB (~15% of the 10 million cases in 2015) (7, 8), Xpert's routine use as a frontline test in those with previous TB (20 to 30% of presumptive TB patients in South Africa) (9), and Xpert-based active case finding in people with previous TB (10).

This issue is compounded by the imminent introduction of a new, highly sensitive version of Xpert called the Xpert MTB/RIF Ultra test (Ultra) that, by virtue of its superior limit of detection (11), is likely confounded by residual mycobacterial DNA to a greater extent than the current-generation Xpert cartridge. For example, as described in a recent World Health Organization policy document, Ultra's preliminary specificity (relative to the results of culture) was lower in patients with a history of TB than in those with no history of TB, and this specificity reduction was greater than that seen with Xpert (−5.4% [95% confidence interval {CI}], −9.1 to −3.1%) versus −2.4% [95% CI, −4.0 to −1.3%] (12). The significance, however, of supposedly false-positive results is not well understood. Furthermore, the persistence of PCR positivity in the absence of culturable bacilli may predict relapse or subclinical disease (13, 14); however, few studies have monitored Xpert-positive patients after treatment completion.

Previously, in a retrospective evaluation using biobanked sputa from patients with presumptive TB, we described the frequency and predictors of Xpert false positivity in previously treated patients (2). We also showed that Xpert detects DNA from nonintact cells, suggesting that dead bacilli contribute to the false positivity. However, as culture is a suboptimal reference standard (15) and adjunct diagnostic tools like chest radiography have poor specificity, especially in high-risk populations infected with HIV (16, 17), we could not discriminate whether Xpert-positive, culture-negative patients were true Xpert false positives in the absence of prospective follow-up and further investigations. In the current study, we performed repeat microbiological testing, bronchoscopy, and prospective clinical follow-up in a new cohort of patients with previous TB to discern the characteristics, profiles, and frequencies of patients with Xpert-positive, culture-negative results. We also present the results of new analyses performed using pooled data from both our new cohort and a previous study (2).

MATERIALS AND METHODS

Participant recruitment. We consecutively recruited adults (age, ≥18 years) from April 2015 to June 2016, irrespective of whether they were symptomatic, from Cape Town, South Africa, who were previously diagnosed to be sputum Xpert positive in diagnostic research studies at the University of Cape Town (18, 19). Patients had been successfully treated for TB in the program. Through the details provided previously, patients were contacted and asked to return for enrollment in this study. Thus, all enrolled patients had been previously treated for proven TB and were recontacted for opportunistic sampling. We excluded patients currently on treatment. This study was approved by the University of Cape Town Faculty of Health Sciences Ethics Committee (approval 698/2014).

Diagnostic tests, procedures, and clinical follow-up. At recruitment, patients gave two induced sputum specimens (20) and received a previously validated morbidity score, administered by a research nurse (21). One sputum specimen was randomly selected for testing with Xpert, and the other was used for both Ziehl-Neelsen smear microscopy and liquid culture with the MGIT 960 system. Prior to the addition of the Xpert sample reagent, the viscosity of the Xpert sputum was subjectively graded (as mucopurulent, purulent, mucosalivary, or salivary) as previously described (22). At the first visit, patients who tested Xpert positive and culture negative were referred for a clinical examination prior to bronchoscopy. We recorded the semiquantitative level of Xpert-positive results, which are automatically reported by the GeneXpert software on the basis of predefined cycle threshold (C_T) values (C_T values are inversely proportional to the concentration of mycobacterial DNA in a specimen) (23, 24). At examination, patients underwent another round of sputum-based testing with Xpert and culture, as well as chest radiography. Irrespective of the test results from this prebronchoscopy visit, all Xpert-positive, culture-negative patients from enrollment were asked to undergo bronchoscopy with bronchoalveolar lavage as previously described (25). Testing by Xpert and culture was done on the bronchoalveolar lavage fluid (BALF). Patients who were culture positive from any specimen were referred to the program for treatment and exited the study. Patients who initially retested Xpert false positive were visited at home or contacted telephonically in December 2016 to ascertain their health status.

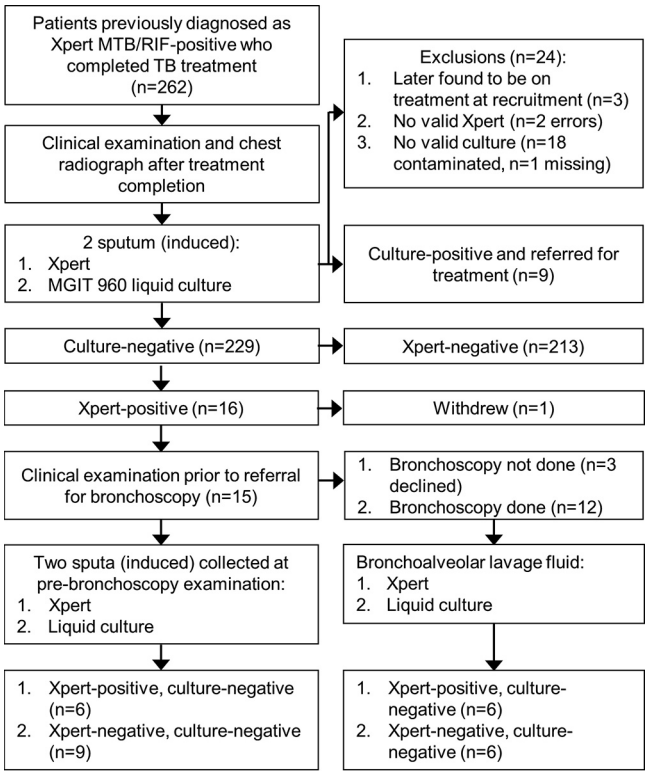

FIG 1 Study flow diagram and test results.

Statistical analyses. We defined patients who were Xpert positive, culture negative using sputa from their first visit as false positive. The χ^2 and Mann-Whitney tests were used to compare the proportions and differences in nonparametric continuous data, respectively. McNemar's test was used to calculate the changes in sensitivity and specificity after reclassification of Xpert-positive results on the basis of their semiquantitative levels. All specificity calculations were done using the paired Xpert and culture result from the first visit (culture served as a reference standard). To analyze how Xpert specificity changed over time since previous treatment completion, we constructed fractional polynomial prediction plots locally weighted to the number of Xpert-negative, culture-negative (true-negative) patients at each time point. Exact binomial 95% confidence intervals were calculated. We used a sliding time window (e.g., at 2 months, only patients for whom 2 months or longer had passed since the completion of treatment for their previous case of TB were included in the specificity calculation). To achieve greater statistical power for analyses involving years, we combined our data with those from a previously described cohort of patients with a history of TB who were symptomatic and underwent Xpert testing (2). We used GraphPad Prism (version 6.0; GraphPad Software) and Stata (version 14; StataCorp) software. All statistical tests were two-sided at an α level of 0.05.

RESULTS

Xpert-positive results in previously treated TB patients. Of the 262 patients recruited, 24 were excluded (Fig. 1). A median of 11 months (interquartile range [IQR], 5 to 19 months) passed between the completion of treatment for the previous episode and recruitment. As determined using sputa collected at recruitment, 9 of the 238 (4%) included patients were culture positive and 5/9 (55%) were Xpert positive. Sixteen of 229 (7%) culture-negative patients were Xpert positive (specificity, 93%; 213/229 [95% CI, 89 to 96%]). False-positive patients had a lower bacillary burden (mycobacterial DNA) than true-positive patients (median Xpert C_T, 28.66 [IQR, 27.21 to 30.4] versus 17.64 [16.86 to 18.20]; $P < 0.001$), an increased likelihood of a chest radiograph not compatible with active TB (5/15 patients versus 0/5 patients; $P = 0.026$), sputum that was less viscous (15/16 patients versus 2/5 patients had a grade of mucoid or less; $P = 0.038$), and trends toward more recent previous TB (5 months [IQR, 5 to 6 months] versus 11 months [IQR, 4 to 16 months] since treatment completion; $P = 0.112$) and a lower morbidity score (0 [IQR, 0 to 1] versus 3 [IQR, 3 to 4]; $P = 0.067$). Internal positive control C_T values, used to detect possible PCR inhibition (26), did not differ between

false- and true-positive patients (24.8 [IQR, 22.4 to 26.1] versus 25.4 [IQR, 24.5 to 26.2]; $P = 0.561$). Six of the 16 Xpert-positive, culture-negative patients (38%) met WHO symptom criteria for TB testing (Table 1) (10).

Repeat Xpert and culture results in false-positive patients with a history of TB. Fifteen false-positive patients were successfully referred for clinical examination (median time since recruitment, 2 months [IQR 2 to 3 months]) prior to bronchoscopy. Xpert and culture were repeated with sputum, and the number of Xpert-positive patients decreased to six (nine were now Xpert negative). We detected no significant differences, including the time between repeat testing (2 months [IQR, 2 to 2 months] versus 2 months [IQR, 1 to 2.25 months]; $P = 0.723$), between patients whose Xpert result remained positive and those whose Xpert result changed to negative. All 15 patients were still sputum culture negative. Of the 12 patients who underwent bronchoscopy (Fig. 1; three patients declined bronchoscopy), 6 patients were BALF Xpert positive and 6 were BALF Xpert negative (all 12 were BALF culture negative). The results of Xpert with BALF had a poor concordance with the results of Xpert with sputum collected at the prebronchoscopy clinical examination visit: half of sputum Xpert-positive patients were BALF Xpert negative (3/6), and half of BALF Xpert-positive patients were sputum Xpert negative (3/6). An overview of each false-positive patient, their clinical characteristics, and their results are provided in Table 1.

Clinical follow-up. Of the 16 Xpert false-positive patients, 15 were successfully followed up after a median time of 17 months (IQR, 16 to 18 months) (patient RX102 relocated). Fourteen patients were reported to be healthy and asymptomatic, including the 3 (patients RX59, RX122, and RX146) who opted out of bronchoscopy. One patient (patient RX124; a heavy smoker) reported a persistent cough but had recently been screened for TB at the local clinic and was Xpert and culture negative.

Period since previous TB and Xpert false positivity. Of the 229 culture-negative patients, 214 (94%) had data on the number of months that had passed since their previous TB treatment. A total of 172/214 (80%) had treatment within 24 months, including 15 false-positive patients (1 false-positive patient was missing this information). Hence, when the data for culture-negative patients who had received treatment within the last 2 years were excluded (Fig. 2A), the specificity rose from 93% (95% CI, 89 to 96%; 213/229) to 100% (95% CI, 92 to 100%; 42/42).

As our cohort of retested patients included those with relatively recent TB, to explore the impact of time since previous treatment on a larger scale (years), we did new analyses with data pooled with data from another cohort of patients with a history of TB (2). Using these pooled data, the overall specificity in patients with previous TB was 94% (95% CI, 93 to 95%; 951/1,011), and the specificity increased to 95% (95% CI, 94 to 96%; 974/920) and 97% (95% CI, 95 to 98%; 689/713) when the data for patients who had completed treatment within 1 and 2 years, respectively, were excluded from calculations (Fig. 2B). The specificity plateaued thereafter.

We next evaluated whether the time (in years) since previous treatment completion could accurately classify false-positive patients in our pooled data (Fig. 3). This could be useful to channel patients into different diagnostic algorithms for those with a recent history of TB that account for Xpert's compromised specificity. If a same-year cutoff point were used, 30% (95% CI, 24 to 35%; 52/203) of Xpert false-positive patients would be correctly classified, increasing to 52% (95% CI, 46 to 58%; 106/203) and 64% (95% CI, 58 to 70%; 130/203) when cutoff points of ≤ 2 and ≤ 3 years, respectively, were used. However, high proportions of patients with active TB who would be correctly detected by Xpert would be erroneously excluded as likely false positive (83% [95% CI, 82 to 85%; 1,315/1,582], 67% [95% CI, 65 to 69%; 1,062/1,582], and 54% [95% CI, 52 to 56%; 854/1,582] for each of the three cutoff points, respectively). The amount of time since the previous treatment alone is, hence, unsuitable for accurately discriminating between patients that are likely to be Xpert false or true positive.

Reclassification of Xpert MTB/RIF-positive patients with a semiquantitative level of very low as Xpert negative. Using pooled data, Xpert's sensitivity and

TABLE 1 Overview of Xpert-positive, culture-negative (false-positive) patients with a history of previous TB[a]

Patient no.	HIV infection status	Morbidity score at recruitment[b]	No. of WHO TB symptoms (symptom type) at recruitment[c]	WHO symptom criteria for active TB investigation met[d]	Chest radiography findings	Sputum Xpert and culture results prior to bronchoscopy	Time (mo) from enrollment to repeat sputum testing	BALF Xpert and culture results
RX059	P	0	1 (current cough)	Yes	Compatible with active TB, right unilateral cavitation	Xpert negative, culture negative	2	Bronchoscopy declined
RX061	N	0	1 (cough for ≥2 wk)	Yes	Compatible with old TB	Xpert negative, culture negative	2	Xpert negative, culture negative
RX065	P	0	0	No	Compatible with active TB, no cavitation	Xpert negative, culture negative	2	Xpert negative, culture negative
RX076	P	0	0	No	Compatible with active TB, no cavitation	Xpert positive, culture negative	2	Xpert positive, culture negative
RX099	P	2	3 (cough for ≥2 wk, wt loss, fatigue)	Yes	Compatible with active TB, bilateral cavitation	Xpert negative, culture negative	2	Xpert positive, culture negative
RX101	P	0	0	No	Compatible with active TB, no cavitation	Xpert positive, culture negative	2	Xpert negative, culture negative
RX102	P	1	0	No	Compatible with active TB, no cavitation	Xpert negative, culture negative	2	Bronchoscopy not done
RX108	N	0	1 (current cough)	No	Compatible with active TB, no cavitation	Xpert positive, culture negative	2	Xpert positive, culture negative
RX115	P	0	0	No	Compatible with active TB, left unilateral cavitation	Xpert negative, culture negative	2	Xpert positive, culture negative
RX122	P	2	3 (wt loss, fever, chest pain)	Yes	Compatible with old TB	Xpert negative, culture negative	2	Bronchoscopy declined
RX124	P	1	1 (cough for ≥2 wk)	Yes	Compatible with old TB	Xpert positive, culture negative	1	Xpert positive, culture negative
RX146	P	0	0	No	Compatible with active TB, no cavitation	Declined further participation after first visit	NA	Bronchoscopy declined
RX502	P	0	0	No	Not done	Xpert positive, culture negative	3	Xpert negative, culture negative
RX512	P	7	6 (cough for ≥2 wk, wt loss, fever, night sweats, fatigue, chest pain)	Yes	Compatible with active TB, no cavitation	Xpert positive, culture negative	1	Xpert negative, culture negative
RX539	P	1	0	No	Compatible with active TB, right unilateral cavitation	Xpert negative, culture negative	2	Xpert negative, culture negative
RX565	N	0	0	No	Compatible with active TB, cavitation present but extent unrecorded	Xpert negative, culture negative	1	Xpert positive, culture negative

[a]All patients were clinically well without treatment after long-term follow-up. Abbreviations: BALF, bronchoalveolar lavage fluid; P, positive; N, negative; TB, tuberculosis; WHO, World Health Organization; NA, not applicable.
[b]The morbidity score refers to the TBscore, which has been described previously (21).
[c]See reference 32.
[d]See references 32 and 33.

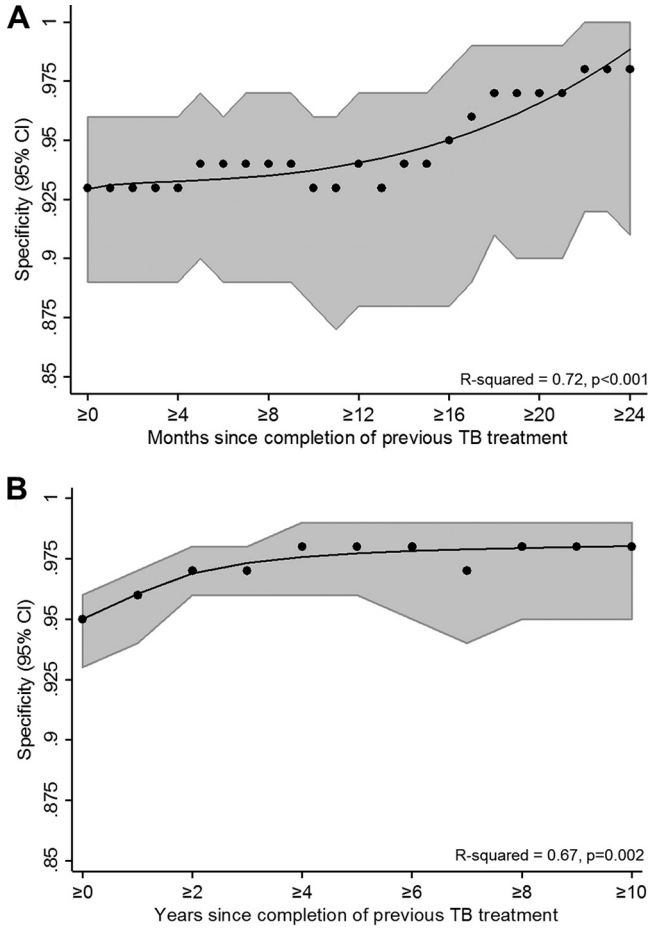

FIG 2 Specificity of Xpert MTB/RIF at different time points, in months (A) and years (B), after the completion of treatment for the previous episode of TB. Sliding windows including patients at each time point or thereafter were used to calculate specificity. When the analyses were restricted to patients without recent previous TB, the specificity improved.

specificity in previously treated patients were 84% (95% CI, 80 to 88%; 326/388) and 93% (95% CI, 88 to 96%; 984/1,049), respectively, whereas after reclassification they were 75% (95% CI, 70 to 79%; 289/388; $P = 0.001$) and 97% (95% CI, 96 to 98%; 1,020/1,049; $P < 0.001$), respectively. The specificity hence improved (+3% [95% CI, +2 to +5%]), but sensitivity was reduced (−10% [95% CI, −4 to −15%]).

DISCUSSION

This study is the first to undertake a prospective follow-up and an exhaustive investigation to correctly classify Xpert-positive, culture-negative results in patients with previous TB. The key findings are as follows: (i) false-positive Xpert results occur in a significant minority of patients with previous TB, (ii) Xpert-positive, culture-negative patients remained healthy (or had minimal symptoms) and were consistently culture negative without treatment, suggesting that they do not have active disease, (iii) although the characteristics of false-positive patients differ significantly from those of true-positive patients, these characteristics (most importantly, the time since previous TB) have a poor predictive capability for identifying patients likely to be false positive, and (iv) Xpert-positive results in previously treated patients were transient, and about half transitioned to Xpert-negative results after later retesting.

We have previously described the increased likelihood of Xpert false-positive results in symptomatic patients with previous TB; however, here we show for the first time that this can occur in patients who are healthy or have minimal symptoms. In a routine

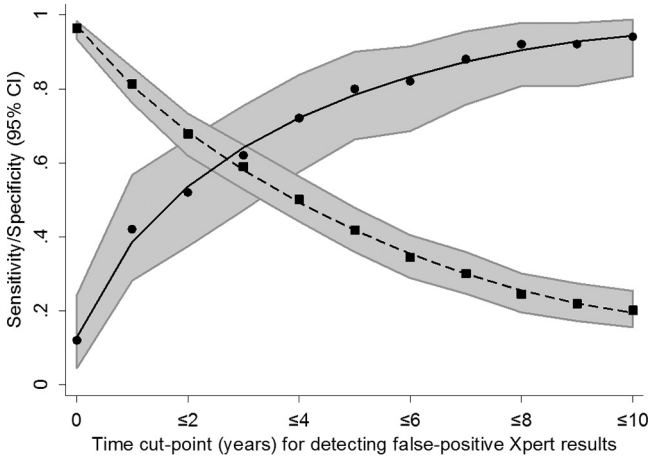

FIG 3 Suboptimal sensitivity (squares, dashed line) and specificity (circles, solid line) of different cutoff points (years) for classifying patients with previous TB as likely to be false or true positive with Xpert.

context, such individuals may be tested with highly sensitive nucleic acid amplification tests as contacts of index cases or as part of targeted active case finding in special populations. Xpert has been reported to have a high specificity in the community-based screening of healthy individuals; however, this was in a setting with a very low prevalence of previous TB (27). This requires confirmation in settings with a high burden of patients with previous TB.

Importantly, we found that the amount of time since previous treatment was likely not useful to programs or clinicians looking to accurately discriminate between patients with a history of TB who are likely to be Xpert false positive and those who are true positive. Although the decreasing likelihood of Xpert false positivity since the time since previous treatment has been described before (2, 3), here we describe this phenomenon for the first time on a fine scale (i.e., months), which is important, as most recurrences occur within 1 to 2 years of treatment completion and the use of this more granular time scale may be more sensitive than the use of a blunter years-based approach (28, 29). This finding regarding time is similar to our previous findings regarding the mycobacterial load (C_T value), where, although patients with false-positive results had significantly fewer bacilli in their sputum than patients with true-positive results, this did not translate into clinically useful predictive values (2). Interestingly, we also found patients with false-positive results to have less-viscous sputum. This is congruent with our findings regarding C_T values, as more salivary sputum has previously been shown to be associated with a lower bacillary load (30).

Importantly, Xpert false positivity was transient. After an average of 2 months between recruitment and repeat sputum testing (prior to bronchoscopy), over half of Xpert false-positive patients were Xpert negative. Thus, detection of DNA from a previous TB episode can be stochastic (this may be due to sampling error or the gradual elimination of pathogen DNA by the host). HIV-positive patients, even those on antiretroviral therapy, have compromised pulmonary immune clearance (31) and may thus be more susceptible to sustained false positivity.

After long-term follow-up, false-positive patients remained culture negative and asymptomatic without treatment, indicating that they were highly unlikely to go on to develop active TB. This is despite the fact that most false-positive patients, including those with HIV infection, had a chest radiograph compatible with active TB. This complicates the management of Xpert-positive patients with prior TB. Thus, in our cohort of retested patients with a history of previous TB and minimal symptoms, all Xpert-positive, culture-negative patients overall had little evidence of active TB after an intensive workup.

We found that reclassifying Xpert false-positive patients with the lowest semiquan-

titative level as negative increased the specificity. However, the resultant reduction in sensitivity was more than 2-fold the specificity gained. This may limit the attractiveness of this approach, which has been mooted for the new Ultra's trace level. Such a reclassification in Ultra may result in changes in sensitivity different from those in Xpert (12); however, the payoffs should be modeled in different patients and settings.

In terms of limitations, first, at the time of our study, Ultra was not available, and its specificity requires investigation in patients with previous TB. Our findings also require validation in different populations, settings, and studies containing larger sample sizes. Second, we targeted known previous TB cases irrespective of their symptoms. Although many had symptoms upon retesting, they are a population distinct from self-presenting patients with prior TB. It is also possible that patients who are Xpert positive after treatment completion might still benefit from treatment, despite being culture negative (e.g., to reduce relapse); however, this requires prospective investigation.

In conclusion, these findings have implications for the interpretation of Xpert-positive results in patients with previous TB and provide evidence of Xpert's suboptimal specificity. It also informs how clinicians should manage false-positive patients. Importantly, given that clinical and test characteristics (including the C_T values) and the time since previous treatment cannot accurately classify which patients will likely have a false-positive Xpert result, careful clinical assessment and additional tools (e.g., culture, clinical follow-up) remain essential for the management of Xpert-positive patients with previous TB.

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G.T. and K.D. conceived and designed the study. All authors collected the data. G.T. performed the analysis and prepared the first draft. All authors interpreted the findings and provided important intellectual input.

We have no conflicts of interest to declare.

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Appendix III

Bacterial and host determinants of infectiousness in patients with drug-resistant versus drug-susceptible tuberculosis

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Bacterial and host determinants of infectiousness in patients with drug-resistant versus drug-susceptible tuberculosis

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Abstract

A burgeoning epidemic of drug-resistant tuberculosis now threatens to derail global tuberculosis control efforts. Although the mechanisms remain poorly clarified, drug-resistant strains are widely believed to be less efficient at airborne transmission and hence less infectious than drug-susceptible strains. We hypothesised that multiple factors, including mycobacterial genomic variation, would be associated with infectiousness. We quantified infectiousness using a cough aerosol sampling system that enumerated *Mycobacterium tuberculosis* colony forming units in respirable (≤10µm) cough-generated aerosols from 500 tuberculosis patients (227 with drug-resistance), performed mycobacterial whole genome sequencing, dormancy phenotyping, and drug susceptibility analyses, and compared clinical characteristics. Even after taking into account treatment duration, we found that almost half of drug-resistant tuberculosis patients were highly infectious, challenging current beliefs. Surprisingly, neither mycobacterial genomic variants, lineage, nor dormancy status predicted high infectiousness. Several mycobacterial and clinical characteristics, including minimal symptoms and stronger cough, were associated with infectiousness, supporting a change in public health strategy to one of active rather than passive case finding. Effective treatment largely abrogated infectiousness but some patients remained infectious despite effective treatment. These data question current paradigms, inform public health strategies to reduce tuberculosis transmission, and suggest the need to redirect tuberculosis transmission-associated research efforts towards host-pathogen interactions.

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Drug-resistant tuberculosis (TB), an underdiagnosed disease characterised by high mortality, unsustainable costs, limited effective drugs, and severe treatment-related adverse events, is predominantly caused by airborne transmission rather than reactivation or acquired drug resistance.[1-5] Animal[6-9], epidemiological[10-18], and modelling studies[19,20] all indicate that an infectious minority of patients drive TB epidemics. Understanding and identifying these sentinels of transmission – sometimes termed "super-spreaders" – and rendering them non-infectious is critical for epidemic control.[19,21] There are, however, few data about the infectiousness of patients with drug-resistant TB, which might be associated with the differential fitness and transmission potential of strains[23,24]. We defined infectiousness as the combined ability of the index case and pathogen to generate infectious quanta measured using *Mycobacterium tuberculosis* colony forming units from inhalable cough aerosol[22]. We hypothesised that drug-resistant and drug-susceptible patients would exhibit large variation in infectiousness. We reasoned this would be explained by host and mycobacterial factors, including those known fitness-encoding mutations[25], given that these genomic variations would likely underpin the ability of more infectious strains to better withstand the environmental stress of aerosolisation, dehydration, and airborne travel compared to less infectious ones. We also evaluated the effect of treatment on infectiousness, which has implications for transmission interruption interventions.

We therefore undertook the largest study to date of inhalable cough aerosols (**Supplementary Figure 1**) from adult drug-susceptible and drug-resistant pulmonary TB patients (n=500 total). This approach used a cough aerosol sampling system (CASS) (**Figure 1A**) that enumerates and size-classifies aerosol particles with culturable *Mycobacterium tuberculosis*.[22,26,27] We collected comprehensive clinical data that included symptom score, HIV status, lung function, radiographic disease severity[28], and treatment history. Microbiological information included sputum Xpert MTB/RIF, Nile-Red staining of dormancy-associated intracellular fatty acids[29],

smear microscopy, MGIT960 culture, sequencing and phenotypic drug susceptibility testing[30] (**Online Methods**). We categorised patients into Groups 1 (non-rifampicin resistant; n=227), 2 (rifampicin-resistant but not to fluroquinolones and aminoglycosides; n=162), or 3 (extensively drug-resistant; resistance to rifampicin, fluroquinolones and aminoglycosides; n=109). We defined "CASS-positives" as patients with any *M. tuberculosis* aerosol colony forming units (CFUs) and a drug as "likely effective" if *in vitro* resistance was undetected. We re-sampled CASS-positive patients and separately assessed reproducibility.

Compared to Groups 1 and 2, Group 3 patients had more disease on chest radiography, more previous TB episodes (including more previous drug-resistance episodes), worse lung function [peak expiratory flow (PEF), forced expiratory volume (FEV_1)] and, despite more TB drugs, received fewer likely effective drugs and were on treatment longer before CASS (**Supplementary Tables 1-2**). Of 452 patients with reliable (non-contaminated) CASS results, 142 (31%) were CASS-positive (**Figure 1B**). 42/142 (30%) of CASS-positives had ≥10 CFU in aerosol (reflecting high infectiousness) and 60% of patients' CFU was in particles ≤4.7 μm likely to be deposited in the smallest airways[31] (**Figure 1C**). 24/34 (70%) of Group 2 and 9/23 (40%) of Group 3 CASS-positives were outpatients, reflecting risk of community-based transmission. Although a small number of smear-negatives were CASS-positive [9/137 (7%)], aerosol CFU positively correlated with sputum bacillary load (**Figures 1D-E**). We then calculated the diagnostic accuracy of different biomarkers for infectiousness using CASS as a reference standard (**Supplementary Table 3**). Receiver operator characteristic areas under the curves (smear grade, C_{Tmin}; **Figure 1F**) were similar and, at rule-out thresholds (≥95% sensitivity), specificities were suboptimal (42% for smear, 33% for Xpert). At rule-in thresholds (≥95% specificity), sensitivities were poor (14%, 15%).

A greater proportion of Group 1 patients were CASS-positive than in Groups 2 and 3 combined [82/201 (41%) vs. 60/249 (24%); p<0.001] (**Figure 1G**). After stratification by treatment

duration, similar proportions of patients on treatment ≤48h were CASS-positive in Group 1 vs. Groups 2 and 3 combined [38/73 (52%) vs. 18/48 (48%); p=0.116] and the median (IQR) aerosol CFU from CASS-positives did not differ [4 (2-16) vs. 2 (1-20); p=0.757]. CASS-positivity was more frequent in patients on treatment for shorter durations [e.g., Group 1: 28/73 (53%) ≤48h vs. 86/329 (26%) >48h, p<0.001; **Supplementary Table 4**] and aerosol CFU exhibited a similar pattern (**Supplementary Figure 3**). However, some patients posed an infectious risk despite likely effective treatment for several days and adherence per records (**Supplementary Tables 5 and 6**). For example, 7/27 (26%) of Group 1 patients treated for eight days were CASS-positive.

Surprisingly, neither lineage spoligotype nor *M. tuberculosis* genomic variants (SNPs, indels) were associated with CASS-positivity (**Figure 2A; Supplementary Results**). Nonsynonymous *rpoB* and *pks1* variants trended towards an association but did not reach significance. Similarly, genomic analyses for sputum culture time-to-positivity (itself a predictor of CASS-positivity[26] and transmission[32]) did not detect associations (**Supplementary Figure 4**). Furthermore, clusters comprised of ≥1 CASS-positive isolate(s) had similar inter-isolate single nucleotide polymorphism distances and number of linkages to other isolates compared to clusters comprised exclusively of CASS-negatives (**Supplementary Table 7**). Nile-Red straining did not detect an association with CASS-positivity (**Figure 2B**).

In unadjusted univariate analyses (**Table 1**), CASS-positivity was associated with younger age, male sex, smoking, being community-based, fewer symptoms, no HIV infection, not receiving TB treatment, better lung function (PEF, FEV_1), higher peak cough flow (PCF), mucopurulent sputum, sputum bacillary load, and drug-susceptibility (patient group). However, in multivariate analyses that considered treatment duration but not individual drugs **(Table 1)**, fewer symptoms, stronger PCF and increased sputum bacillary load independently-predicted CASS-positivity. Patients with HIV or in Group 2 were less likely to be CASS-positive.

Similarly, in a clinically-orientated model including data only available from rapid tools, Xpert C_{Tmin} and treatment ≤48h independently-predicted CASS-positivity. When these variables were incorporated into a clinical prediction rule, the rule had higher AUC than C_{Tmin} alone; correctly classifying approximately double the proportion of CASS-positives [32% vs. 15%, p<0.001)] (**Figure 2F, Supplementary Table 2**). Although pulmonary cavitation was weakly associated with sputum bacillary load and CASS-positivity (**Supplementary Figure 4**), the latter relationship was absent in multivariate analyses.

We examined the association of drug regimen with CASS-positivity. In univariate analyses, CASS-positivity was inversely associated with each additional drug in the regimen [OR (95% CI) 0.75 (0.65, 0.87) per drug; p<0.001] and a fluoroquinolone (FQ)-based regimen [0.32 (0.19, 0.53); p<0.001]. The latter was the only drug-related variable significant in a multivariate model [0.34 (0.17, 0.65); p=0.001] (**Supplementary Tables 8-9**).

66/80 (83%), 29/34 (85%), and 18/23 (78%) baseline CASS-positive patients in Groups 1, 2, and 3, respectively were re-sampled at least once (**Figure 3)**. 3/66 (5%) in Group 1 were repeat CASS-positive despite likely effective treatment and adherence (one Group 2 repeat positive non-adherent). Group 3 patients displayed prolonged infectiousness with 7/18 (40%) repeat CASS-positive. In a separate cohort of pre-treatment TB patients(n=29; **Supplementary Tables 10-11**), CASS was most reproducible across morning samplings ("fair"[33] CASS-positivity kappa, "substantial"[34] aerosol CFU intraclass correlation coefficient).

Our two main findings are that almost half the patients with drug-resistant TB (including community-discharged therapeutically destitute patients) are highly infectious within the first 48h of treatment, and there is a lack of association between infectiousness and mycobacterial genomic variants. Other key findings were that 1) mycobacterial physiological state was similar in CASS-positives versus negatives, 2) specific host factors (fewer symptoms, stronger cough,

no HIV, treatment ≤48h) and mycobacterial factors (higher sputum bacillary load) independently predict infectiousness, 3) a subset of patients had elevated infectiousness during likely effective treatment (although more pronounced for drug-resistant TB, this was also observed for drug-susceptible TB), and 4) fluoroquinolones were associated with reduced infectiousness.

The transmissibility of differentially drug resistant strains is controversial[23,35]. We have now shown that drug-resistant patients are highly infectious based on culturable aerosol production. We hypothesised that lineage and genomic variants influence infectiousness; however, we found no such linkages. This suggests that there are alternative post-aerosolisation mediators of strain success, including host genetic background, strain epigenetics, inhalational burden reaching alveoli, and repetitive infection events. Sputum with high Nile-Red positivity is associated with reduced culturability[39,40] but we did not detect associations with CASS-positivity. Alternative methods of measuring dormancy, like those that use resuscitation-promoting factors[36], might be more sensitive.

We identified new host factors that overwhelmingly drove infectiousness, in addition to those described earlier.[27,37] Our finding that healthier patients are more infectious support rollout of community-based active case finding as a public health strategy that targets cases with minimal symptoms. The advent of portable molecular diagnostics makes this approach feasible[38].

Patients with drug-sensitive TB are often considered non-infectious approximately two weeks after effective treatment initiation[39-41] and animal models suggest that, even within 48h, drug-resistant cases may be non-infectious[42]. By contrast, we found culturable aerosol in 34% of drug-susceptible cases on likely effective treatment >48h. Although we did not recruit drug-susceptible cases on treatment beyond two weeks, a low proportion of drug-susceptible CASS-positives (4%) were still detected a month after re-sampling, suggesting that a persistently

infectious drug-susceptible subset exists. This may be because of suboptimal pharmacokinetics, including due to poor cavitary penetration[43], heteroresistance, or undocumented treatment non-compliance. These data support cautious approaches to decide when TB patients are non-infectious.[41,44,45] Indicative of few treatment options available at the time, when baseline Group 3 CASS-positives were re-sampled, 40% remained CASS-positive – including some over a year since treatment initiation. This underscores the need for scale-up of new drugs, and provision of community-based palliative and long stay facilities[46,47]. Our inability to establish an accurate clinical prediction rule suggests the need for a user-friendly test for infectiousness.

A strength and limitation of our approach is that we measured infectiousness (transmission potential) rather than transmission itself, which is a complex multifactorial process[48]. Indeed, we could not measure the effects of inhalation, alveolar germination, and host immunity all of which animal models can do[9,42,49-55]. However, CASS-measured cough aerosol culturability is the biomarker most highly associated with transmission-related outcomes (e.g. skin test conversion and active TB) in humans.[27,56,57] Low infectiousness (CASS-negativity) may still result in transmission if exposure is prolonged, repetitive, or occurs in poorly ventilated areas. Characterising *M. tuberculosis* in aerosol, especially in its *de novo* physiological state, is challenging and we hence interrogated Nile Red status, however, this measures only one dimension of dormancy.

In conclusion, drug-resistant TB is highly infectious. Mycobacterial genomic variation and physiological state were not associated with infectiousness. Increased infectiousness among patients with minimal symptoms supports the urgent need for community-based active case finding approaches, and new infection control strategies as patients can remain infectious despite prolonged effective treatment.

Data availability statement

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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Figure legends

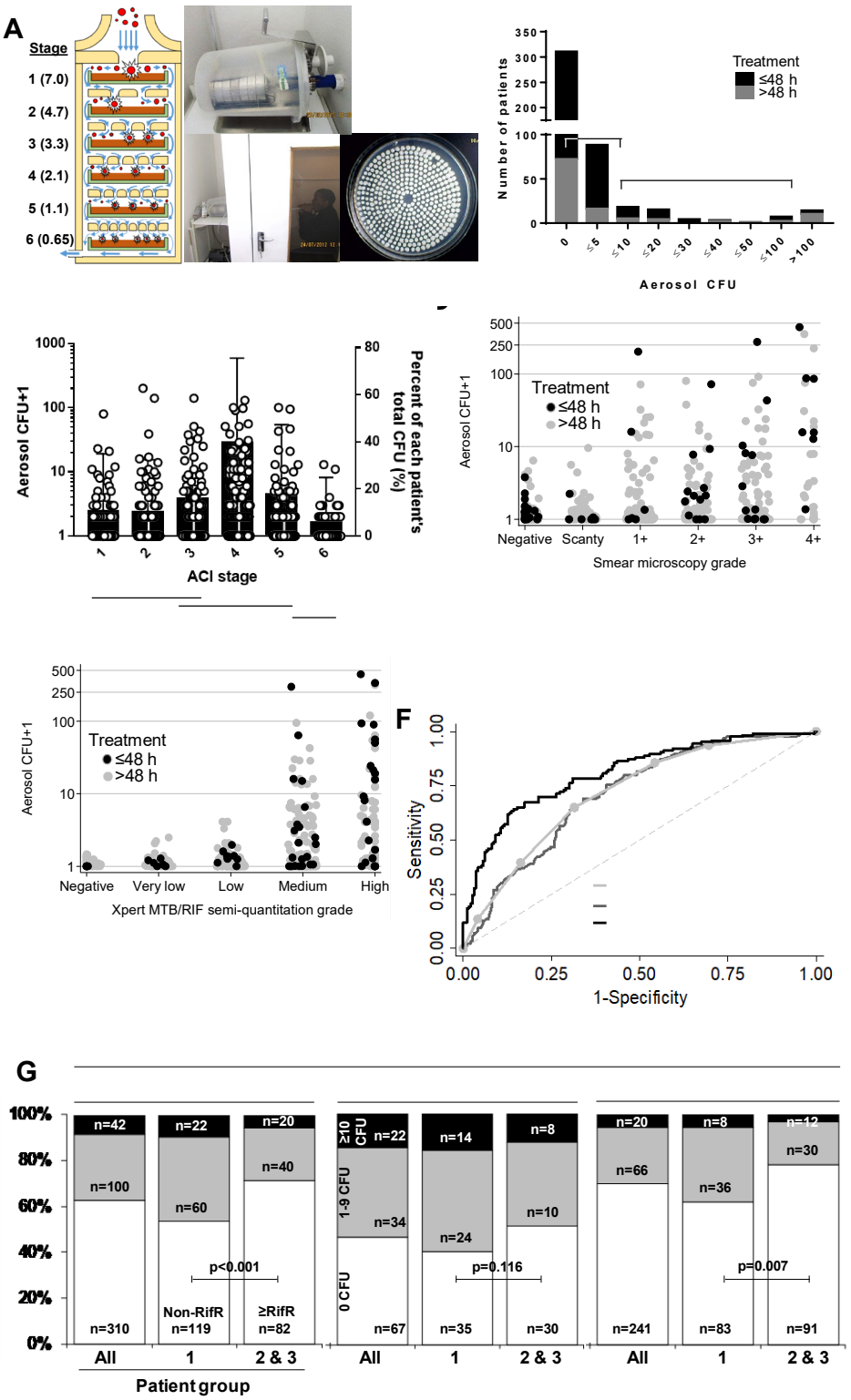
Figure 1. A cough aerosol sampling system (CASS) was used to measure infectiousness **(A)** consisting of (i) a six-stage Anderson Cascade Impactor [median expected droplet diameter (µm) for each stage is shown] that (ii) lies horizontally in a 10 l autoclavable chamber that (iii) patients cough into (iv) permitting CFU from individual aerosol droplets to be isolated in a size-dependent manner. **(B)** Most patients (n=310) were CASS-negative and a minority of CASS-positives with varied infectiousness (1-310 CFU) were identified. **(C)** CFU counts were highest in the 2.1-4.7 µm particle size range (stages 3-4) (circles, left y-axis), and, on average, most patients' total CFU came from particles 2.1-3.3 µm in diameter (stage 4) [bars (standard deviation, right y-axis]. CFU in aerosol correlated with **(D)** sputum smear microscopy grade and **(E)** Xpert MTB/RIF semi-quantitation grade [both D and E have the proportion of CASS-positive patients and median (IQR) aerosol CFU in CASS-positives indicated], and **(F)** these translated into moderate-to-high receiver operator characteristic curve areas under the curves (AUCs) in analyses for CASS-positivity (shown for sputum Xpert MTB/RIF-positive patients) that were improved by a clinical prediction rule. **(G)** CASS-positivity is shown by patient group and treatment status (all patients, treatment ≤48 h, treatment >48h). Black, grey, and white indicate the proportions of patients with >10, 1-9, or 0 aerosol CFU. P-values are for comparisons of Group 1 vs. Groups 2 and 3 within patients of the same treatment status. After adjustment for treatment status, no differences in infectiousness by drug susceptibility were detected (Table S5 provides additional data). B, D-E: black indicates no treatment or on treatment ≤48 h, grey indicates treatment >48 h. C and D have logarithmic axes and one was added. A is adapted from [58]. Abbreviations: AUC, area under the curve; CASS, cough aerosol sampling system; CFU, colony forming units; CI, confidence interval; IQR, interquartile range; RifR, rifampicin resistant.

Figure 2. Comparison of mycobacterial factors in CASS-positive and negative patients. **(A)** Manhattan plot showing that whole genome sequencing of sputum culture isolates identified no variants associated with CASS-status that met significance thresholds (n=115 CASS-positives, n=203 CASS-negatives). The top variant was in *rpoB* (a S531L mutation known to cause rifampicin resistance). The dashed lines above panel represent the p-value threshold for significant associations. Analyses sub-stratified by treatment status, HIV status, and different CASS CFU cut-points reached similar conclusions. **(B)** A representative image of Auramine and Nile-Red strained sputum showing a mixture of Auramine-positive bacilli (green) with or without Nile-Red co-localisation (red; left panel) and a dot plot (mean, standard deviation) of the proportion of Auramine-positive bacilli also Nile-Red positive. Additional analyses by CASS status after sub-stratification by patient group (i.e., drug susceptibility) and/or treatment status revealed no differences.

Figure 3. Repeat cough aerosol sampling results in patients initially CASS-positive who were re-sampled until CASS-negative. Most Group 1 **(A)** or Group 2 **(B)** patients were CASS-negative at repeat sampling after treatment (the only Group 2 patient with persistent CASS-positivity was non-adherent) but many Group 3 patients were repeatedly CASS-positive **(C)**. Each line represents a patient and each symbol a sampling. Microbiology results (microscopy, Xpert MTB/RIF, culture) from paired sputa are below the panels and continued to have poor diagnostic accuracy for CASS-positivity. Patients CASS-positive more than once are thick black lines. Patients not on treatment are dashed lines. Triangles in (C) indicate patients discharged with programmatically uncured drug-resistant TB (three baseline CASS-positives of this type and not on treatment were CASS-negative at re-testing). Vertical dashed lines indicate the median days to the second and third CASS. Y-axes are logarithmic and one was added to CFU counts. Abbreviations: ACI, Andersen Cascade Impactor; CASS, cough aerosol

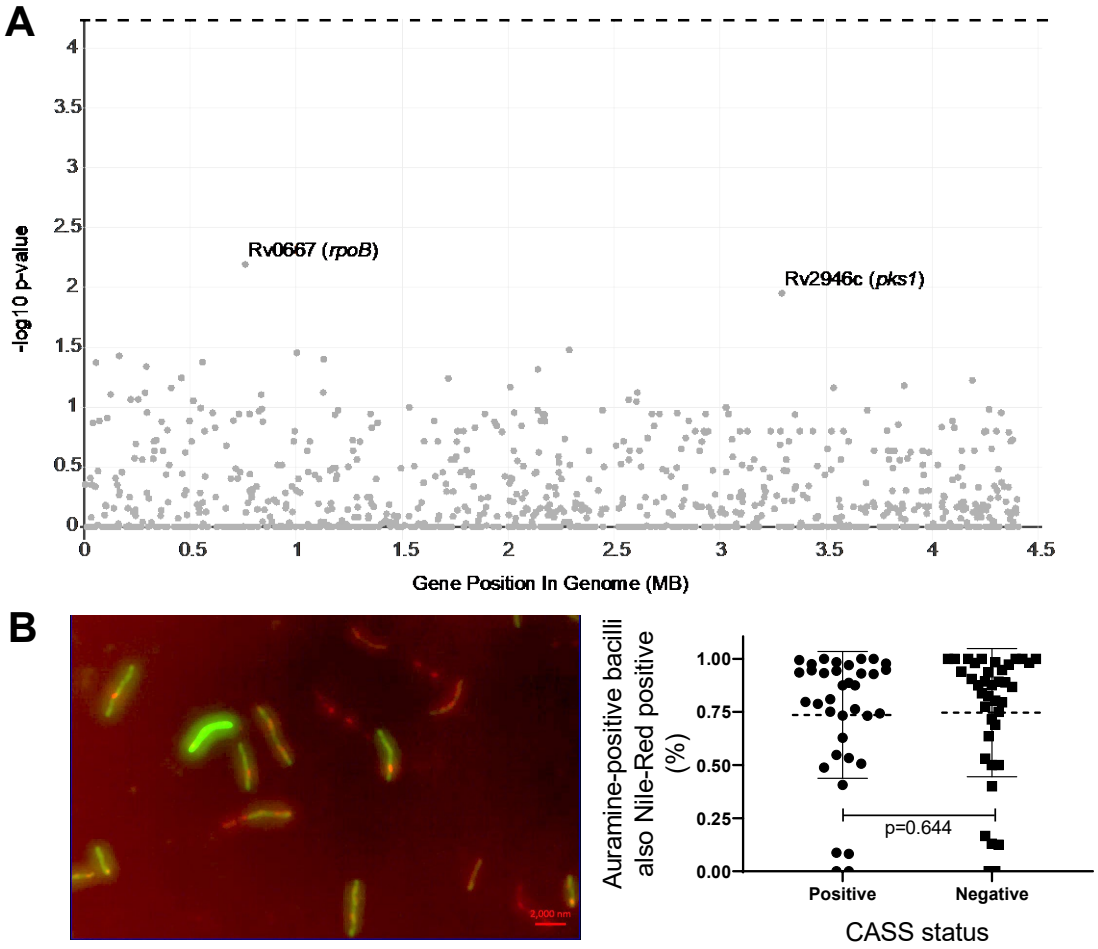
413 sampling system; CFU, colony forming units; MDR, multidrug resistant; IQR, interquartile
414 range; Xpert, Xpert MTB/RIF; XDR, extensively drug-resistant.

415 **Figure 1.**



Missing data: Smear microscopy grade (n=2); Xpert MTB/RIF grade (n=46).
B: 16 of the 142 (11%) CASS-positives had CFU exclusively in particles corresponding to ≥4.7 µm, however, 10/16 (63%) had at least three plates discarded due to the contamination.

416 **Figure 2.**



417

418 **Figure 3.**

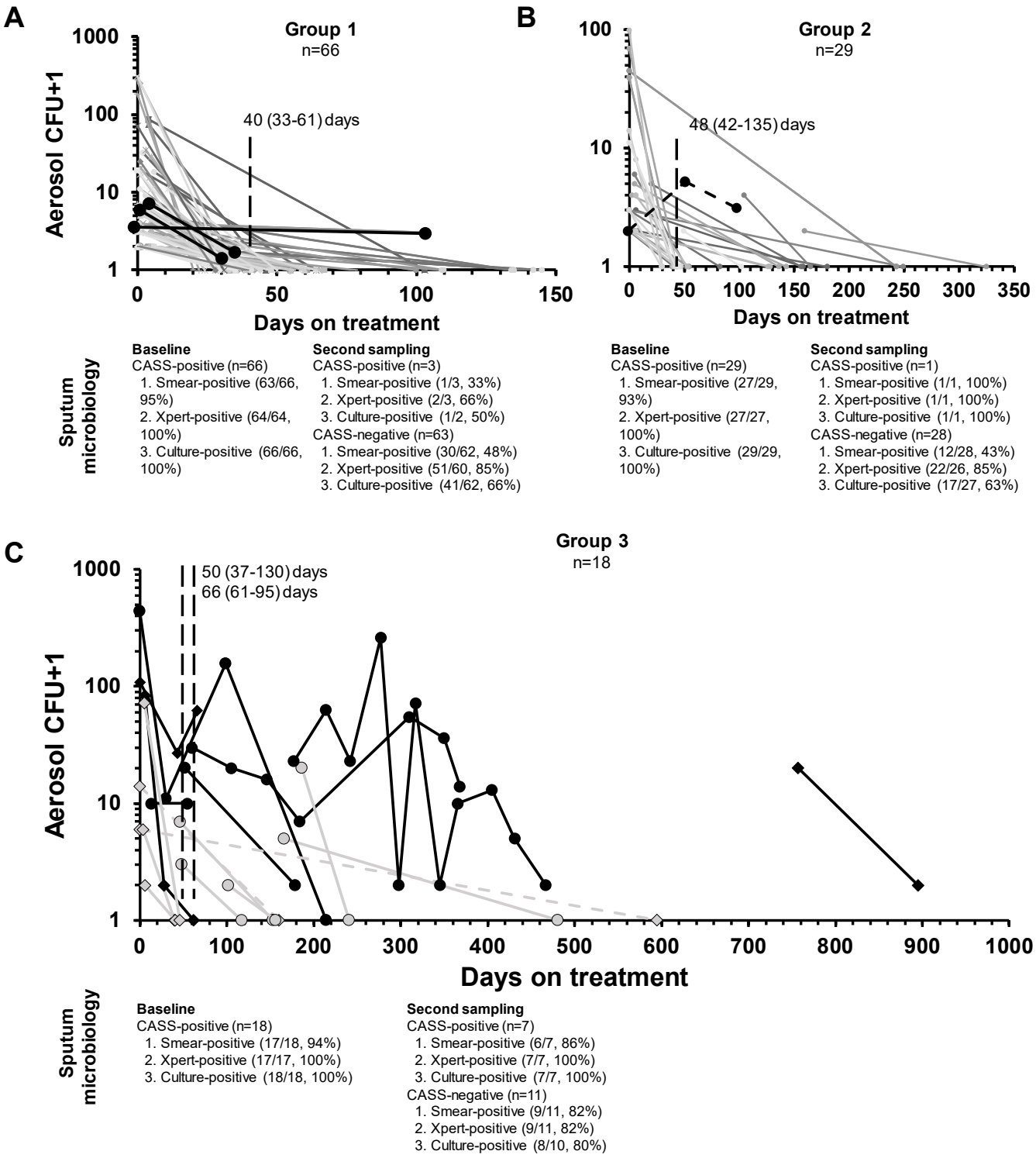


Table 1. Univariate and multivariate logistic regression analyses of predictors of cough aerosol culture-positivity. When analyses were repeated for only patients on treatment >48h **(Supplementary Tables 7-8)**, the same predictors remained significant. Data are median (IQR) or n/N (%) unless otherwise stated.

	Univariate analyses (n=452)				**Multivariate analyses**[†]			
					Sputum culture-positive n=254		**Sputum Xpert MTB/RIF-positive** n=377	
	CASS-negative (n=310)	**CASS-positive** (n=142)	**Unadjusted odds ratio** (95% CI)	**P-value**	**Adjusted odds ratio** (95% CI)	**P-value**	**Adjusted odds ratio** (95% CI)	**P-value**
Demographic characteristics								
Age, years	35 (28-45)	33 (27-43)	9.83 (9.65, 10.00) (per 10 years)	**0.061**	-	-		
Female sex	137/308 (45)	43/142 (30)	0.54 (0.36, 0.83)	**0.004**	-	-		
Smoker (current or previous)	184/308 (60)	100/142 (70)	1.59 (1.03, 2.43)	**0.034**	-	-		
Based in community	195/308 (63)	117/142 (83)	2.69 (1.65, 4.38)	**<0.001**	-	-		
Clinical characteristics								
TB symptom score	4 (2-5)	3 (2-5)	0.91 (0.63, 1.00) (per unit)	**0.054**	0.78 (0.68, 0.90) (per unit)	**0.001**	0.79 (0.69, 0.91) (per unit)	**0.001**
HIV-positive	142/307 (46)	41/141 (29)	0.48 (0.31, 0.74)	**0.001**	0.41 (0.23, 0.75)	**0.003**	0.55 (0.32, 0.96)	**0.034**
On TB treatment >48 h[§]	241/308 (78)	77/142 (54)	0.33 (0.21, 0.50)	**<0.001**	-	-	0.54 (0.31, 0.94)	**0.030**
Prior TB	167/308 (54)	67/142 (47)	0.75 (0.51,1.12)	0.165	-	-	-	-
CXR disease extent								
Bilateral[*]	208/276 (75)	100/130 (77)	1.09 (0.67, 1.78)	0.732	-	-	-	-
Disease score	7 (4-10)	7 (5-9.25)	1.02 (0.96, 1.09) (per unit)	0.448	-	-	-	-
Any cavitation	99/276 (36)	66/130 (51)	1.84 (1.21, 2.81)	**0.004**				
Cavitation score	0 (0-1)	0.25 (0-1)	1.17 (0.93, 1.48) (per unit)	0.174	-	-	-	-
Lung function								
PEF (l/min)	165 (58-252)	223 (43-280)	1.22 (1.04, 1.40) (per 100 units)	**0.014**	-	-	-	-
PCF (l/min)	300 (240-363)	330 (280-397)	10.05 (10.02, 10.07) (per 10 units)	**<0.001**	10.08 (10.04, 10.11) (per 10 units)	**<0.001**	10.07 (10.03, 10.10) (per 10 units)	**<0.001**
FEV_1 (l)	1.2 (0.46-1.70)	1.49 (0.65-1.86)	1.42 (1.06, 1.91) (per unit)	**0.018**	-	-	-	-
Aerosol sampling								
Cough count	83 (62.5-107)	85.5 (66-110)	1.03 (0.99, 1.11) (per 10 units)	0.259	-	-	-	-

Ambient conditions								
Room temperature (°C)	19.3 (17.8-22.1)	20 (18-22.3)	1.04 (0.97, 1.11)	0.260	-	-	-	-
Humidity	61 (56-69)	62.5 (55-72)	1.01 (0.99, 1.03)	0.335	-	-	-	-
Sputum microbiology								
Nile-Red positive bacilli (in patients with a sputum smear grade ≥2+), mean proportion (SD)	0.76 (0.30)	0.80 (0.59)	1.24 (0.41, 3.81)	0.701				
Sputum viscosity								
Salivary/mucosalivary	188/259 (73)	73/124 (59)	Reference	N/A	-	-	-	-
Purulent/mucopurulent	71/259 (27)	51/124 (41)	1.86 (1.19, 2.91)	**0.007**	-	-	-	-
Smear grade					Omitted in favour of time-to-positivity		Omitted in favour of C_{Tmin}	
Negative	127/306 (42)	91/142 (6)	Reference	N/A	-		-	
Positive	179/306 (58)	133/142 (94)	10.49 (5.14, 21.38)	**<0.001**	-		-	
SC	43/306 (14)	10/142 (7)	3.28 (1.25, 8.61)	**0.016**	-		-	
P+	54/306 (18)	28/142 (20)	7.31 (3.24, 16.54)	**<0.001**	-		-	
P++	42/306 (14)	35/142 (25)	11.76 (5.22, 26.47)	**<0.001**	-		-	
P+++	29/306 (10)	40/142 (28)	19.46 (8.50, 44.55)	**<0.001**	-		-	
P++++	11/306 (4)	20/142 (14)	25.65 (9.45, 69.69)	**<0.001**	-		-	
Xpert-positive	252/287 (87)	136/136 (100)	Non-calcuable	**<0.001**	Omitted in favour of time-to-positivity		N/A	N/A
C_{Tmin}	21.6 (17.7-26.2)	17.3 (14.7-19.8)	0.84 (0.80, 0.88) (per unit)	**<0.001**	-		0.80 (0.76, 0.86)	**<0.001**
Culture-positive	253/297 (85)	141/142 (99)	24.58 (3.35, 180.38)	**0.002**	N/A	N/A	Omitted in favour of C_{Tmin}. Culture unavailable for rapid decision making	
TTP (days)	12 (8-16)	8 (5-10)	0.80 (0.76, 0.86)	**<0.001**	0.78 (0.72, 0.84)	**<0.001**	-	-
Strain family (spoligotype)								
Bejing	159/245 (65)	86/138 (62)	Reference	N/A	-	-	-	-
LAM	30/245 (12)	18/138 (13)	1.06 (0.55, 2.03)	0.821	-	-	-	-
T	35/245 (14)	18/138 (13)	1.54 (0.50, 4.74)	0.368	-	-	-	-
X	4/245 (2)	2/138 (1)	0.52 (0.51, 1.79)	0.890	-	-	-	-
Other	7/245 (3)	7/138 (5)	1.40 (0.50, 5.01)	0.267	-	-	-	-
Unknown	10/245 (4)	7/138 (5)	0.90 (0.16, 5.02)	0.651	-	-	-	-
Drug-resistance category								
Group 1	119/308 (39)	82/142 (58)	Reference	N/A	Reference	N/A	Reference	N/A
Group 2	112/308 (36)	36/142 (25)	0.47 (0.29, 0.75)	**0.001**	0.48 (0.26, 0.90)	**0.023**	0.45 (0.25, 0.82)	**0.010**
Group 3	77/308 (25)	24/142 (17)	0.45 (0.26, 0.77)	**0.004**	0.57 (0.27, 1.21)	0.144	0.51 (0.25, 1.01)	0.053
Group 2 and 3 combined	119/308 (37)	60/142 (42)	0.46 (0.31, 0.69)	**<0.001**	Omitted in favour of individual groups (1, 2, or 3).			

421 *Patients with a normal CXR were pooled with those with unilateral disease for the comparison with patients with bilaterial disease.
422 †Values as in the final models.
423 §Due to the small number of patients not on any treatment whatsoever, a treatment duration cut-point of 48h used.

Abbreviations: CASS, cough aerosol sampling system; CXR, chest radiography; IQR, interquartile range; PEF, peak expiratory flow; FEV, forced expiratory volume; SC, scanty; SD, standard deviation; C_{Tmin}, minimum cycle threshold value; TTP, time-to-positivity.

Online Methods

Participant recruitment

We identified adult outpatients (≥18 years) through a programmatically-generated list of Xpert MTB/RIF- (Xpert)- or smear-positive patients at clinics in Cape Town received regularly from the National Health and Laboratory Service in Cape Town, South Africa from February 2013 to July 2017. We prioritised recruitment of rifampicin-resistant patients. We had no restrictions based on TB history or HIV status but excluded patients with haemoptysis or unable to expectorate two sputa. Patients were typically newly-diagnosed and not on treatment or had recently started treatment (regimens were determined programmatically). If a newly-diagnosed patient in the community was unavailable, we recruited inpatients with second-line drug-resistance at Brooklyn Chest Hospital (BCH), a specialised provincial drug-resistant TB hospital, where the CASS facility was installed or, using a prospective registry[1], programmatically home-discharged patients with uncured drug-resistant TB. At BCH, we favoured the recruitment of inpatients who were recently admitted or had a recent positive culture-result. Overall, we aimed to recruit at least 100 patients per patient group (defined by drug-resistance profile), expecting 20-30% in each group to be CASS-positive.

Study profile

Consenting patients underwent a nurse-administered clinical examination, including symptom severity (TBscore)[2], HIV-testing, and chest radiography (radiographs were read in a blinded standardised manner by a trained reader[3]; **Tables 1-2**). Peak expiratory flow (PEF), forced expiratory volume (FEV_1) and peak cough flow (PCF) were measured using an Asma-1 Electronic Respiratory Monitor (Vitalograph, United Kingdom) and a Respi-Aide Peak Flow Meter (GaleMed, China). The average of three consecutive blows were used. All procedures were done at one visit in the same order.

Sputum microbiology

Patients expectorated two sputa, one of which was arbitrarily selected for Xpert (Cepheid) and the other NALC-NaOH decontaminated before double Ziehl-Neelsen concentrated smear microscopy[4] and one MGIT 960 liquid culture (BD, United States). Patients needed at ≥1 positive smear (including scanty) to be classified as smear-positive. When gradings were discrepant, the highest was used. The viscosity of the sputum used for Xpert was graded by appearance[5] and an aliquot used for Auramine O-Nile Red staining to detect dormancy-associated intracellular lipid bodies.[6] Culture isolates were confirmed as positive for acid-fast bacilli (AFB) and *M. tuberculosis* complex (MTBDR*plus*, Hain Lifesciences) and used for minimum inhibitory concentration determination using Sensititre MYCOTB plates (Thermofisher, United States; **Table 3**).[7]

Sputum Auramine-Nile Red staining

We used a modified version of previously-described protocols.[6,8] Briefly, a NALC-NaOH decontaminated sputum was centrifuged and resuspended in 125 µl phosphate buffer. 30 µl of the resuspension was mixed with 10 µl fixative (National Health Laboratory Service) and the mixture smeared on a slide before fixation using a heating block at 80 °C for 2 h. In parallel, an additional 125 µl phosphate buffer was added to the resuspended decontaminated sputum (in order to minimise background fluorescence seen in some specimens) and the same procedure followed using both undiluted and diluted slides. Once heat fixed, the slide was sprayed with Mercofix (Merck, United States). Prior to visualization, the slide was flooded with Auramine O (National Health Laboratory Service, South Africa) for 15 min, rinsed with water, decolourised with 0.5% (v/v) acid alcohol, rinsed again, flooded with Nile Red solution (Sigma-Aldrich, South Africa; 10 µg/ml in phosphate buffered saline), rinsed again, flooded with 0.1% (w/v) potassium permanganate for 1 min, rinsed a final time and dried. Three drops of Mowiol mounting agent with DABCO (Merck, United states) were added and slides

visualised within 24 h using an 880 LSM confocal microscope (Carl Zeiss, Germany) and Zen 2011 software (Carl Zeiss, Germany). Stained slides were stored in the dark at 4 °C before visualisation. We counted bacilli with intact Auramine O-positive cell walls as positive for *M. tuberculosis* up to a maximum of 100 per slide. After Auramine O-positive bacilli were identified, we switched to the red-light channel and counted the proportion with Nile Red-positive co-localisation. We encountered significant levels of Nile Red-positive background that made it difficult to consistently distinguish individual cells. After consultation with an imaging specialist, we only counted Auramine O-positive bacilli as Nile Red-positive if the intensity of the red light at that location was at least 50% higher than the background red. Each slide was independently counted by at least two trained readers and the average proportion reported. If readers' counts differed by ≥20%, a third reader was used and the average of the two closest counts reported. We only did Nile Red staining on sputum due to concentrations of bacilli in aerosol below the limit of detection.

Cough aerosol sampling

Procedure

The CASS[9] consists of a six-stage Andersen Cascade Impactor (ACI; Thermofisher, United States) within a 10 l chamber (the maximum autoclavable size in our laboratory). CASS collects aerosol in a particle size-dependent manner. Each ACI stage holds 7H11 solid agar plates (Middlebrook, United States) supplemented with OADC (Middlebrook) and Selectatab (Kircher, United States). CASS was done before sputum expectoration, typically by late morning, using a modified version of a previously-described procedure.[9] Briefly, patients coughed as forcefully and as frequently as possible into the CASS for 5 min via a 1 m silicone pipe that ran from the patient in a sputum induction booth into the 10 l chamber. Ambient temperature and humidity and the number of coughs were recorded. If patients were CASS-positive, they underwent repeat CASS and sputum sampling until CASS-negative. For all

CASS samplings (as with all other clinical and microbiological methods except smear microscopy), measurements were done in a discrete fashion and the same patients or samples were not measured repeatedly.

Biosafety

The apparatus was autoclaved after each use and the plates loaded in a class-2 biosafety cabinet. chamber. The 1 m pipe patients coughed into which was chemically-disinfected and autoclaved after each use. A disposable mouthpiece was used. After passing through the ACI, air was drawn through high efficiency HEPA filters (Whatman, United States) by a pump (Thermo Fischer, United States) at 28 l/min. Flow rate was monitored using a flow meter (Dri Cal, United States). After completion, CASS parts were double-bagged, transported to the Stellenbosch University Biosafety Level-3 facility, dissembled and autoclaved. If patients had to expectorate sputum during CASS, they first turned away from the sampling pipe.

Microbiology

Plates from the ACI were incubated for 10 weeks at 37 °C and checked weekly for growth from week four and AFB-positive CFU counted. Plates overgrown with non-acid fast contaminants were discarded. Patients with contaminated aerosol had unknown CASS status (method of designation as contaminated below).

Classification of agar plates as contaminated

Agar plates from the ACI were checked for *M. tuberculosis* CFU (off-white, grey, crinkled and Ziehl-Neelsen acid fast bacilli positive) for eight weeks. The presence and morphology of any other growth were recorded. Plates with non-*M. tuberculosis* growth, which most frequently appeared fungal, were transferred to a separate container. These plates were subsequently checked for *M. tuberculosis* CFU weekly until a lawn of overgrowth was seen. Plates were discarded prior to eight weeks if overgrown. The median (IQR) time until *M. tuberculosis* CFU detection was 28 (25-39) days. We classified patients as having contaminated aerosol if 1) no

M. tuberculosis CFU were observed and 2) at least three (half of their total plates) were discarded prior to the median time to *M. tuberculosis* CFU detection (28 days). Such patients could not be confidently classified as CASS-positive or –negative.

Reproducibility sub-study

We used a modified version of a previously-described protocol.[9] We did two CASS samplings per day (at least 4 h apart) on two consecutive days and counted the number of CFU from the ACI from each sampling. We recruited patients (n=29) independent of the parent study. Patients typically expectorated sputum during the CASS, however, if they did not, they were asked to expectorate sputum after CASS. Sputa underwent smear microscopy and culture or Xpert (if only one sputum was available, it was used for smear and culture). To improve chances of CASS-positivity, we recruited patients with high sputum bacillary load (at least smear-grade 2+ positive or Xpert semi-quantitation level "medium"), and had been on treatment less than 48 h. Fleiss kappa values were used to compare dichotomous test results and intraclass correlation coefficients were assessed for continuous data.

DNA extractions, genotyping, and whole genome sequencing

DNA for spoligotyping and whole genome sequencing (WGS) was extracted from sputum culture isolates.[10]

Sequence analysis

Processing, alignment and single nucleotide variant calling

Whole-genome sequencing was done using Illumina HiSeq2500. Library preparation was done using the Illumina Genomic DNA Sample Preparation Kit to achieve paired-end sequencing (2×101 bp) with an average insert size of 500 bp. The data processing pipeline used has been described previously[1]. Briefly, we mapped raw sequence data uniquely to the corrected H37Rv reference genome (Genbank accession AL123456.3) using bwa-mem (v0.7) software. We used

148 SAMtools (v1.3) and GATK (v3.3-0) to call single nucleotide polymorphisms (SNPs) and
149 indels. We selected the intersection of variants with quality scores of Q30 or higher, equating
150 to one error per 1000 base positions for further analysis. Genotypes were called in positions of
151 ten-fold coverage; otherwise positions were classified as missing. Highly repetitive and
152 variable regions were removed by calculating mappability values along the reference genome
153 using a k-mer length of 50 base pairs and 0.04% of allowed substitutions while mapping. 364
154 of the 394 (93%) culture-positive isolates from patients with a valid CASS status had DNA
155 was extracted and sequencing was attempted.

156 *Genome-wide association studies*

157 Genome wide associations (GWAS) were computed using R[11] (v 3.3.0). After excluding
158 isolated with a high proportion of missing SNP or less than ten-depth coverage, 318/364 (87%)
159 isolates were included in further analyses, including 115 (36%) from CASS-positives.
160 Univariate analysis with binomial phenotypes (response variables) were computed using a
161 Fisher's Exact test. For multivariate analysis of binomial phenotypes, GWAS was done with
162 nsSNPs on a gene level using an efficient mixed model approach with a kinship matrix to adjust
163 for population structure or using a logistic regression with correction for population structure
164 using principal components. We also performed GWAS by investigating phylogenetic
165 convergence: RaxML was used to construct a best scoring maximum likelihood tree and
166 association analysis was done using the R package treeWAS. Similar methods were applied to
167 the presence of indels on a gene level (i.e., if an indel overlapped a gene region, then it was
168 marked as a mutation in a matrix of all genes by all isolates). Only variants where the minor
169 allele frequency was at least 0.1 were investigated. SNP interactions were investigated using
170 the logitFS R-package. Benjamini-Hochberg multiple test correction was applied.

Inter-isolate SNP comparisons

These were done using all SNPs. 326/364 (90%) of isolates were included in these analyses after filtering those with >10% of the genome having less than ten-depth coverage and presumed mixed-infections. An inter-isolate SNP threshold of 5 (0 to 10 in sub-analyses) was used to link similar strains. Low coverage positions were excluded on a per pair basis. Graphs were constructed in R using igraph (v1.2.4.1). Fisher's exact test was used to test the differences between the number of linked isolates by the dependent variable and the number of connections to and from isolates.

Drug susceptibility categorisation

We used a "rule-in" strategy, where if Sensititre MYCOTB plates indicated phenotypic resistance (isoniazid, rifampicin, ethambutol, ethionamide, kanamycin, ofloxacin, p-aminosalicylic acid, rifabutin, streptomycin, cycloserine, amikacin, and moxifloxacin; critical concentrations in **Table 3**), WGS (using a previously-described panel[12] for all drugs expect pyrazinamide and rifabutin, for which different panels were used[13-15]) indicated genotypic resistance or, for rifampicin, Xpert indicated resistance, patients were classified as resistant to that drug. If patients were missing drug susceptibility test (DST) information from the time of recruitment (e.g., culture-negative or -contaminated), we used DST data available from programmatic records generated from a specimen collected within six months of recruitment (Xpert, MTBDR*plus,* MTBDR*sl,* phenotypic MGIT960 DST). If no DST data were still available, we used diagnoses from medical records (listed as DS-, MDR-, pre-XDR-, or XDR-TB).

We first classified patients according to standard definitions as drug-susceptible (susceptible to rifampicin and isoniazid), rifampicin or isoniazid mono-resistant (resistance to one of these drugs), MDR- (resistant to both rifampicin and isoniazid and susceptible to the second line

drugs), pre-XDR (MDR and resistance to fluoroquinolones or the second line injectable agents), and XDR (MDR and resistance to fluoroquinolones and second line injectable agents).[16] To improve sample size and reduce data over-stratification (which compromises precision)[17], we further categorised patients in a manner congruent with World Health Organization-treatment guidelines at the time of the study.[18] Patients with isoniazid mono-resistance were included with drug-susceptible patients (Group 1), those with rifampicin mono-resistance or pre-XDR were included with MDR patients (Group 2), and XDR- patients were allocated to a separate category (Group 3).

Spoligotyping

Decontaminated sputum specimens or MGIT culture aliquots were heated at 100ºC for ≥1 h. Two microliters of lysate was used for spoligotyping PCR (Kamerbeek method[19]). Briefly, PCR was done to selectively amplified the left and central direct repeat target with the internationally standardized PCR protocol in combination with primers DRa (GGTTTTGGGTCTGACGAC, 5′ biotinylated) and DRb (CCGAGAGGGGACGGAAAC).[19] Products were then hybridized to a membrane containing bound oligonucleotides and detected via chemiluminescene on X-ray film. Isolates were assigned a genotype family as described by SITVIT.[20] *In silico* spoligotypes were predicted using SpolPred on sputum culture isolate WGS.[21]

Definitions

"CASS-positive", patients with any *M. tuberculosis* CFU from cough aerosol. A drug was considered "likely effective" if there was documented susceptibility and no evidence of resistance (phenotypic or genotypic). Patients were classified based on antibiograms or clinical records into Group 1 (non-rifampicin resistant), Group 2 (rifampicin resistant and pre-XDRs), and Group 3 (XDRs).[22,23] If a susceptible or resistant phenotypic result was not available

(culture-negative, culture-contaminated, or indeterminate) as well as no WGS (and, for rifampicin, a positive Xpert result), the drug was classified as being of unknown effectiveness.

Statistical analyses

The chi-squared test was used for comparisons between proportions. We used Fisher's exact test with mid-p correction for comparisons between proportions and the Mann-Whitney test was used to compare differences in non-parametric continuous data. Multivariable logistic regression was done to adjust for potential confounding (parameters $p \leq 0.100$ included in models). A backward elimination strategy using the log likelihood ratio test was used to finalise models. Analyses were done using Graphpad Prism (version 6.0; GraphPad Software, United States) and Stata (version 14; StataCorp, United States). All statistical tests were two-sided at $\alpha=0.05$. Continuous data were tested for normality using the D'Agostino-Pearson omnibus test. Adjustments for multiple comparisons were not done.

Ethics

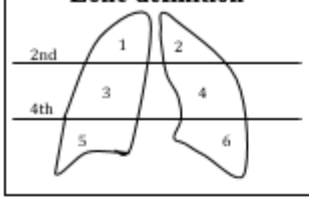
Research ethics committees at the University of Cape Town (038/2008) and Stellenbosch University (N13/01/001) approved the study.

234 **Table 1.** Variables used to calculate TB symptom score.[2]

Parameters	**Points assigned** (Maximum score is 13)
Self-reported	
Cough	1
Haemoptysis	1
Dyspnoea	1
Chest pain	1
Night sweats	1
Clinically examined	
Anaemic conjunctivae	1
Tachycardia	1
Positive finding at lung auscultation	1
Axillary temperature > 37.0 °C	1
BMI < 18.0	1
BMI < 16.0	1
MUAC < 220 mm	1
MUAC < 200 mm	1

235 Abbreviations: BMI, body mass index; MUAC, middle upper arm circumference.

Table 2. Chest radiography scoring system.[3]

Zone definition	
	□ Bilateral disease □ Effusion □ Glands □ Unilateral disease

DATE:						
Zones affected	**1**	**2**	**3**	**4**	**5**	**6**
Disease extent						
Score (a)						
Cavitation						
Score(b)						
Total score(a) + (b)						
↓						
Composite score all zones		.				

Legend	*Symbol*	*Score*
Disease (a)		
No disease	**Leave blank**	**0**
<50% of area affected	**<**	**1**
≥ 50% of area affected	**>**	**2**
Cavitation (b)		
No cavitation	**Leave blank**	**0**
Single cavity, < 2cm diameter	**1a**	**0.25**
Single cavity, 2-4cm diameter	**1b**	**0.50**
Single cavity, > 4cm diameter	**1c**	**1.00**
Multiple cavities, largest < 2cm diameter	**2a**	**0.50**
Multiple cavities, largest 2-4cm diameter	**2b**	**1.00**
Multiple cavities, largest > 4cm diameter	**2c**	**2.00**

239 **Table 3.** Drug range concentrations and the critical concentrations used to define phenotypic
240 resistance with Sensititre MYCOTB plates.[7]

Drug	Sensititre range (µg/ml)	Critical concertation (µg/ml)
Isoniazid	0.03-4	1
Rifampicin	0.12-16	1
Ethambutol	0.5-32	10
Ethionamide	0.3-40	5
Kanamycin	0.6-40	5
Ofloxacin	0.25-32	2
P-aminosalicylic acid	0.5-64	2
Rifabutin	0.12-16	0.5
Streptomycin	0.25-32	10
Cycloserine	2.0-256	25
Amikacin	0.12-16	5
Moxifloxacin	0.06-8.0	2

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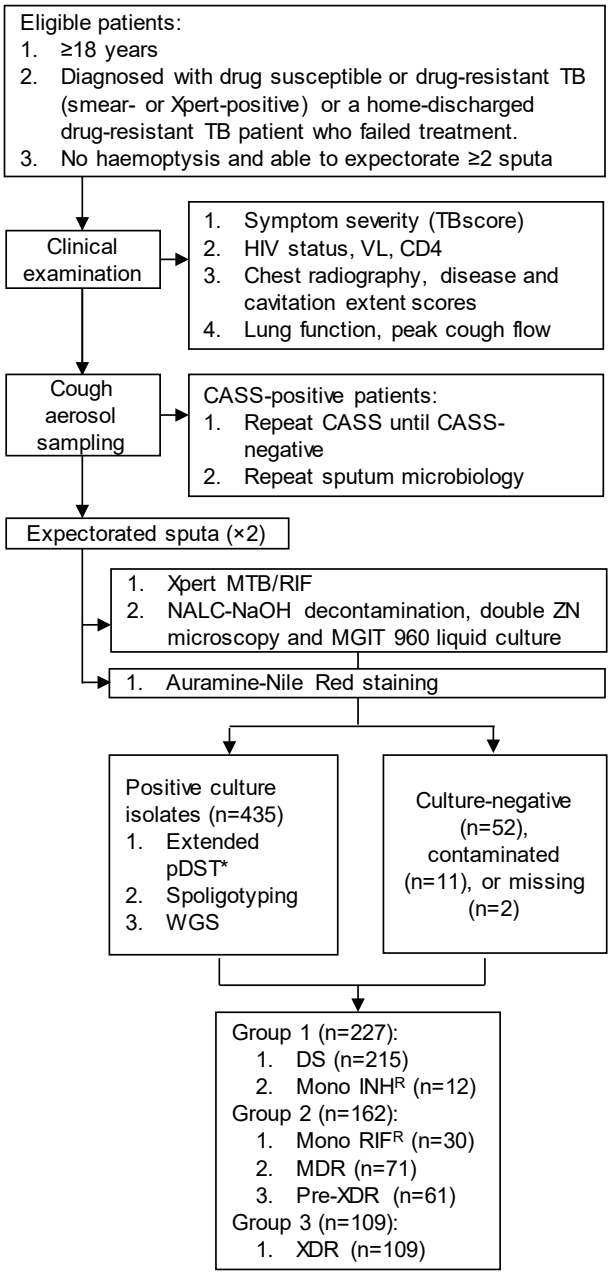
Supplement

43 **Supplementary Figures**



*Done using Sensititre MYCOTB plates for isoniazid, rifampicin, ethambutol, ethionamide, kanamycin, ofloxacin, p-aminosalicylic acid, rifabutin, streptomycin, cycloserine, amikacin, and moxifloxacin

Figure 1. Study profile and participant overview. Abbreviations: CASS, cough aerosol sampling system; DS, drug-susceptible; INH, isoniazid; MDR, multidrug-resistant; MGIT, mycobacterial growth indicator tube; RIF, rifampicin; TB, tuberculosis; XDR, extensively drug-resistant; VL, viral load; WGS, whole genome sequencing.

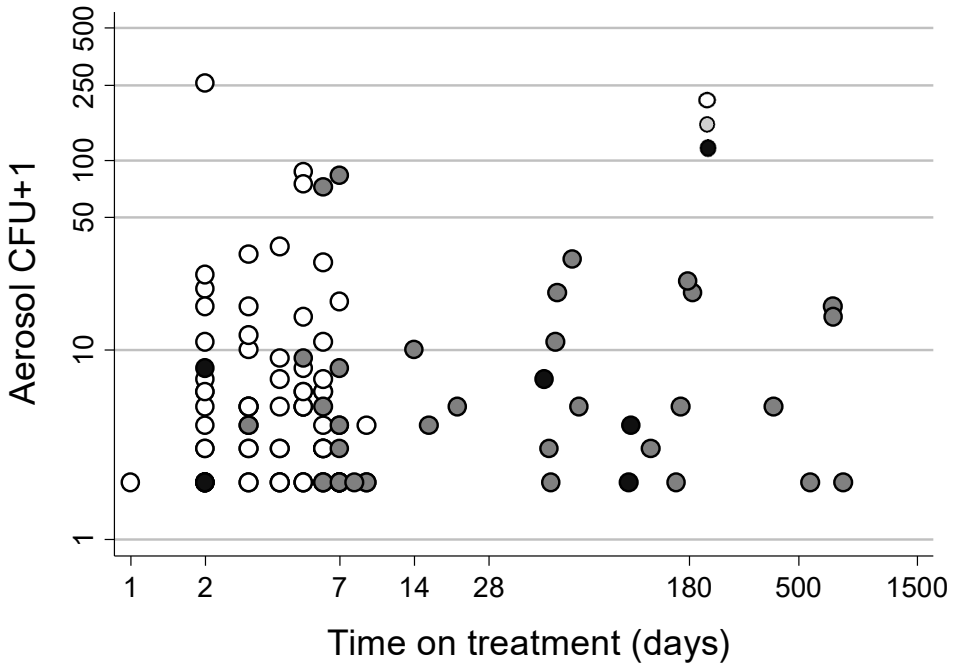


Figure 2. *M. tuberculosis* CFU from cough aerosol particles at recruitment as a function of days on treatment before CASS stratified by regimen type. Beyond eight days, no patients receiving the first-line regimen were CASS-positive, whereas patients receiving second-line regimens had CFU in their aerosol for months. The y-axis is logarithmic and one was added. Abbreviations: ACI, Andersen Cascade Impactor; CLF, clofazimine; FQ, fluoroquinolone; IQR, interquartile range; SLID, second-line injectable drug.

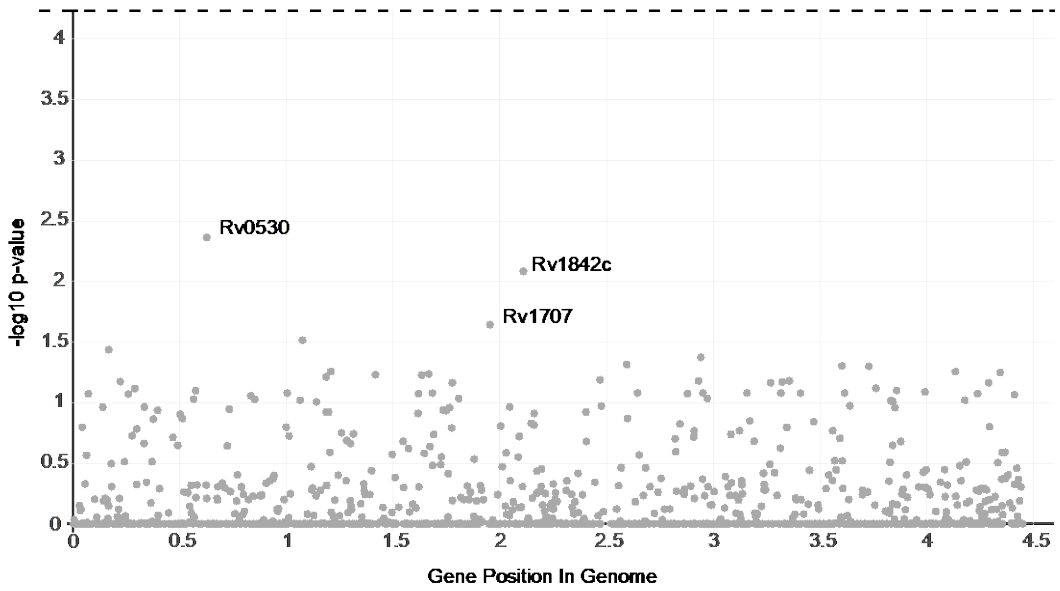


Figure 3. Genome wide association study (GWAS) Manhattan plot of sputum culture time-to-positivity with correction using the first two principal components of the genomic variants.

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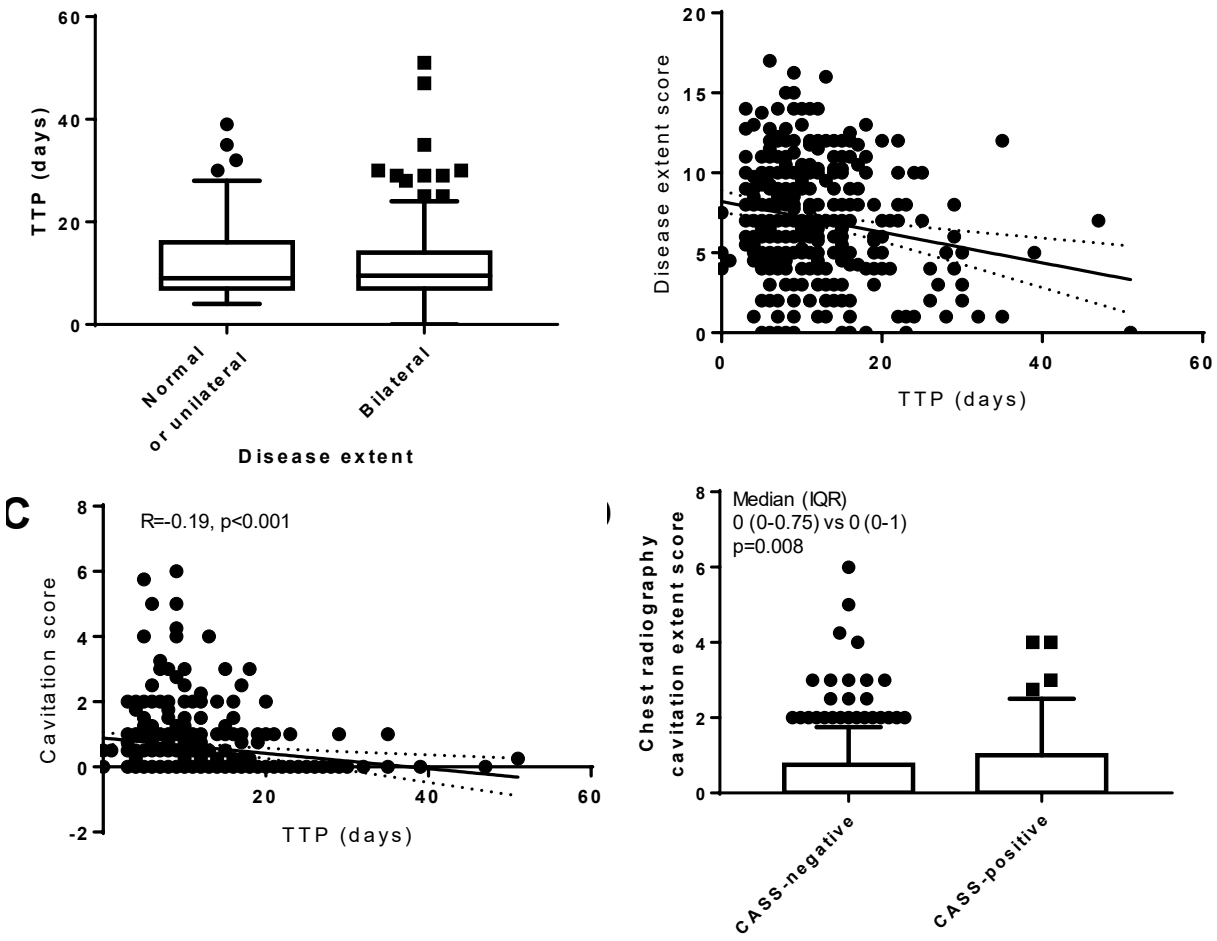

Figure 4. Relationships between sputum bacillary load (liquid culture time to positivity) and disease extent **(A and B)** and cavitation score **(C)**, and between CASS-status and cavitation score **(D)** assessed by chest radiography[1].

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Supplementary Tables

Table 1. Patient characteristics by drug susceptibility. Data are median (IQR) or n/N (%) unless otherwise stated.

	Group 1 (n=227)	**Group 2** (n=162)	**Group 3** (n=109)
	Non-rifampicin resistant	Rifampicin-resistant or Pre-XDR	XDR
Demographic characteristics			
Age (years)	35.4 (27.5-45.8)	32.8 (26.8-42.2)	34.3 (28.7-43.5) (p=0.748)
Female gender	85/227 (38)	70/162 (43)	46/109 (42) (p=0.472)
Smoker (current or previous)	143/227 (63)	93/162 (57)	75/109 (69) (p=0.160)
Location			
In community	227/227 (100)	96/162 (60)	27/109 (25) **(p<0.001)**
In hospital	0	66/162 (40)	82 /109 (75)
Clinical characteristics			
HIV			
Negative	141/226 (63)	88/162 (54)	65/108 (60) (p=0.274)
Positive	85/226 (38)	74/162 (46)	43/108 (40)
ARVs	21/85 (25)	43/74 (61)	35/43 (81) **(p<0.001)**
Viral load	91126 (3982-260000)	3420 (47-130000)	43 (40-34432) **(p<0.001)**
CD4 (cells/µl)	198 (129-347)	217 (84-362)	181 (112-383) (p=0.927)
TB symptom score	3 (2-5)	3 (2-5)	3 (1-5) (p=0.369)
CXR disease extent			
Normal	6/202 (3)	4/147 (3)	1/102 (1) (p=0.772)
Unilateral	46/202 (23)	29/147 (20)	24/102 (24)
Bilateral	150/202 (74)	114/147 (78)	77/102 (76)
Disease score	6.5 (4-9)	7 (5-10)	7 (4-10) **(p=0.026)**
Any cavitation	85/202 (42)	60/147 (41)	41/102 (40) (p=0.944)
Cavitation score	0 (0-1)	0 (0-1)	0 (0-1) (p=0.963)
Previous TB	84/227 (37)	102/162 (63)	66/109 (61) **(p<0.001)**
Prior drug-resistant TB	1/81 (1)	37/100 (37)	18/65 (28) **(p<0.001)**
Lung function			
PEF (l/min)	218 (58-289)	188 (58-248)	164 (114-248) **(p=0.001)**
FEV_1 (l)	1.4 (0.5-1.9)	1.3 (0.5-1.7)	1.2 (0.5-1.67) **(p=0.003)**
Cough strength			
PCF (l/min)	317 (253-373)	307 (250-280)	303 (233-370) (p=0.507)
Sputum microbiology			
Viscosity			
Salivary	64/193 (33)	31/142 (22)	20/93 (22)

			(p=0.129)
Mucosalivary	68/193 (35)	64/142 (45)	46/93 (50)
Purulent	30/193 (15)	27/142 (19)	14/93 (15)
Mucopurulent	31/193 (16)	20/142 (14)	13/93 (14)
Smear microscopy			
Negative	64/225 (28)	51/162 (32)	39/109 (36) (p=0.393)
Positive	161/225 (72)	111/162 (68)	70/109 (64)
Proportion Nile Red positive, mean (SD)*	0.80 (0.59)	0.74 (0.30)	0.77 (0.25) (p=0.959)
Culture			
Negative	20/221 (9)	19/158 (12)	11/106 (10) (p=0.592)
Positive	201/221 (91)	139/158 (88)	95/106 (90)
Strain family (spoligotype)			**p<0.001**
Bejing	92/197 (47)	94/136 (69)	79/91 (87)
LAM	46/197 (23)	10/136 (8)	1/91 (1)
T	28/197 (14)	26/136 (20)	8/91 (9)
X	5/197 (3)	1/136 (1)	1/91 (1)
Other	13/197 (7)	1/136 (1)	1/91 (1)
Unknown	13/197 (7)	4/136 (3)	1/91 (1)
Xpert MTB/RIF			
Negative	17/207 (8)	14/157 (9)	9/105 (9) (p=0.455)
Positive	190/207 (92)	143/157 (91)	96/105 (91)
TB treatment			
On any treatment	201/227 (89)	136/162 (84)	93/109 (89) (p=0.332)
Days treatment for current episode prior to cough aerosol sampling	4 (2-5)	9 (5-96)	72 (35-148) **(p<0.001)**
Regimen			**p<0.001**
First-line	196/198 (99)	4/128 (3)	5/86 (6)
FQ-based	2/198 (1)	115/128 (90)	76/86 (88)
SLID- or CLZ-based (no FQ)	0/198 (0)	9/128 (7)	5/86 (6)
Number drugs received†	4 (4-4)	6 (5-7)	7 (5.5-8) **(p<0.001)**
Likely effective (susceptible)	4 (3-4)	2 (1-3)	2 (1-2) **(p<0.001)**
Likely ineffective (resistant)	0 (0-0)	2 (1-3)	4 (2-5)
Effectiveness unknown§	0 (0-1)	1 (1-3)	1 (1-2)
On treatment >48 h	21/227 (9)	62/162 (38)	30/109 (28) **(p<0.001)**

Missing data or error or contamination: HIV (n=2), TB symptom score (n=16), chest radiograph (n=47), type of previous TB (n=6), smear microscopy (n=9), PEF and FEV_1 (n=27), PCF (n=32), culture (n=13), spoligotype (n=74), Xpert (n=25), sputum viscosity (n=71); regimen (n=18).

*Nile-Red staining not possible in all patients (see supplement).

†The regimens in each patient group are in Table S4.

§For DST, we were unable to determine likely effectiveness for the following drugs: RIF, n=16; INH, n=39; PZA, n=120; EMB, n=62; MOX, n=33; OFL, n=1; KAN, n=15; CAP, n=29; ETH, n=12; PAS, n=33; LNZ, n=3. This was due to isolates that did not regrow, were contaminated, gave indeterminate DST results, or WGS was of insufficient quality to allow accurate SNP calling.

Abbreviations: DST, drug-susceptibility testing; IQR, interquartile range; MDR-, multidrug-resistant; PEF, peak expiratory flow; FEV, forced expiratory volume; INH, isoniazid; RIF, rifampicin; SD, standard deviation; SNP, single nucleotide polymorphism; TB, tuberculosis; XDR, extensively drug-resistant.

Table 2. Individual drugs by patients and likely drug effectiveness, according to drug susceptibility profile (patient group).

	Group 1 (n=201)	**Group 2** (n=136)	**Group 3** (n=97)
RIF	199/201 (99)	4/136 (3)	5/97 (5) **(p<0.001)**
Likely effective	183/183 (100)	0/4 (0)	0/5 (0) **(p<0.001)**
INH	199/201 (99)	100/136 (74)	62/97 (64) **(p<0.001)**
Likely effective	158/170 (93)	14/93 (15)	1/69 (1)
PZA	200/201 (100)	123/136 (90)	91/97 (94) **(p<0.001)**
Likely effective	141/145 (97)	24/81 (29)	28/68 (41) **(p<0.001)**
EMB	200/201 (100)	102/136 (75)	76/97 (87) **(p<0.001)**
Likely effective	170/170 (100)	39/80 (49)	10/66 (15) **(p<0.001)**
MOX	1/201 (1)	111/136 (82)	75/97 (77) **(p<0.001)**
Likely effective	1/1 (100)	58/89 (65)	3/64 (100) **(p<0.001)**
OFL	1/201 (1)	10/136 (7)	3/97 (3) **(p=0.002)**
Likely effective	1/1 (100)	6/9 (67)	0/3 (0) (p=0.084)
AMI	0/201 (0)	2/136 (2)	0/97 (0) (p=0.111)
Likely effective	-	2/2 (100)	-
KAN	2/201 (1)	65/136 (48)	12/97 (12) **(p<0.001)**
Likely effective	2/2 (100)	47/50 (94)	1/12 (8) **(p<0.001)**
CAP	0/201 (0)	49/136 (36)	62/97 (64) **(p<0.001)**
Likely effective	-	25/33 (76)	8/49 (16) **(p<0.001)**
ETH	3/201 (2)	100/136 (74)	60/97 (62) **(p<0.001)**
Likely effective	3/3 (100)	23/92 (25)	8/56 (14) **(p=0.002)**
PAS	1/201 (1)	56/136 (41)	74/97 (76) **(p<0.001)**
Likely effective	1/1 (100)	39/42 (93)	48/55 (87) (p=0.630)
CLOF	0/201 (0)	55/136 (40)	71/97 (73) **(p<0.001)**
Likely effective	-	32/32 (100)	54/54 (100)
LNZ	0/201 (0)	1/136 (1)	5/97 (5) **(p=0.001)**
Likely effective	-	1/1 (100)	2/5 (40) (p=0.273)
TERI	2/201 (1)	120/136 (88)	88/97 (91) **(p=0.001)**
CLA	0/201 (0)	3/136 (2)	2/97 (2) (p=0.111)

Abbreviations: RIF, rifampicin; INH, isoniazid; PZA, pyrazinamide; EMB, ethambutol; MOX, moxifloxacin; OFL, ofloxacin; AMI, amikacin; KAN, kanamycin; CAP, capreomycin; ETH, ethionamide; PAS, p-aminosalicylic acid; CLOF, clofazimine; LNZ, linezolid; TERI, terizidone; CLA, clarithromycin

Table 3. Diagnostic accuracy of smear microscopy grade, Xpert MTB/RIF quantitative information (C_{Tmin}), and a clinical prediction rule for culture-positive cough aerosol in different clinical scenarios (rule-out, rule-in, Youden). Data are % (95% confidence interval).

Test use scenario	Suggested cut-point*	Sensitivity	Specificity	PPV	NPV	Positive LR	Negative LR
	Smear microscopy Grade						
-	Positive	94 (88, 97)	42 (36, 48)	43 (37, 48)	93 (88, 97)	1.61 (1.39, 1.85)	0.15 (0.06, 0.32)
Rule-out	≥Scanty	94 (88, 97)	42 (36, 48)	43 (37, 48)	93 (88, 97)	1.61 (1.39, 1.85)	0.15 (0.06, 0.32)
Rule-in	≥5+	14 (9, 21)	96 (94, 98)	65 (45, 81)	71 (66, 75)	3.94 (1.40, 11.64)	0.89 (0.81, 0.97)
Youden	≥2+	87 (80, 92)	56 (50, 61)	47 (41, 54)	90 (85, 94)	1.96 (1.60, 2.38)	0.24 (0.13, 0.4)
	Xpert MTB/RIF C_{Tmin}						
-	Positive (any C_{Tmin})	100 (97, 100)	13 (10, 18)	35 (30, 0.40)	100 (91, 100)	1.15 (1.08, 1.22)	0 (0, 0.28)
Rule-out	≤24.7	95 (90, 98)	33 (27, 40)	49 (43, 55)	91 (82, 96)	1.43 (1.23, 1.64)	0.15 (0.05, 0.38)
Rule-in	≤13.9	15 (9, 22)	95 (91, 97)	65 (45, 81)	62 (57, 68)	2.71 (0.97, 7.97)	0.9 (0.8, 1)
Youden	≤18.6	66 (58, 74)	74 (68, 80)	63 (55, 71)	77 (70, 82)	2.58 (1.79, 3.75)	0.45 (0.32, 0.63)
	Clinical prediction score $-0.2 \times C_{Tmin} - 0.2 \times$ TBscore $- 1 \times$ HIV $- 1 \times$ TB treatment (>48 h) $+ 1 \times$ (0 if Group 0, -1 if Groups 2 or 3) $+ 0.005 \times$ PCF (l/min) + 3						
Rule-out	-2.95	94 (89, 98)	32 (26, 39)	47 (41, 53)	90 (80, 96)	1.39 (1.6, 1.6)	0.17 (0.44, 0.06)
Rule-in	0.05	32 (24, 41)	95 (91, 98)	80 (66, 90)	68 (63, 74)	6.25 (2.6, 16.51)	0.72 (0.84, 0.61)
Youden	-1.4	71 (63, 79)	78 (72, 84)	68 (59, 76)	81 (75, 86)	3.27 (4.86, 4.86)	0.37 (0.52, 0.25)

*≥95% sensitivity, ≥95% specificity, and the maximum sum of sensivity and specificity (assuming equal weighting) were used to select cut-points for the rule-out, rule-in, and Youden scenarios[2], respectively.

Abbreviations: C_{Tmin}, minimum cycle threshold value; LR, likelihood ratio; PCF, peak cough flow; PPV, positive predictive value; NPV, negative predictive value.

Table 4. Cough aerosol microbiology results by patient group, treatment status and treatment duration. Data are n/N (%) or median (IQR).

	All patients			**No treatment or treatment ≤48 h**			**Treatment >48 h**		
	Overall (n=500)	**Group 1** (n=227)	**Groups 2 and 3** (n=271)	**Overall** (n=132)	**Group 1** (n=77)	**Groups 2 and 3** (n=53)	**Overall** (n=368)	**Group 1** (n=150)	**Groups 2 and 3** (n=218)
CASS-contaminated	48/500 (10)	26/227 (11)	22/271 (8) (p=0.209)*	9/132 (7)	4/77 (5)	5/53 (9) (p=0.349)*	39/368 (11) (p=0.206)†	22/150 (15) (p=0.034)†	17/218 (8) **(p=0.035)*** (p<0.001)†
CASS-negative	310/452 (69)	119/201 (59)	189/249 (76)	67/123 (55)	35/73 (48)	30/48 (63)	241/329 (73)	83/128 (65)	91/201 (45)
Days treatment	6 (4-72)	4 (3-5)	12 (5-116) **(p=0.001)***	1 (1-2)	1 (1-2)	1 (1-2) **(p=0.031)***	8 (5-89)	5 (4-5.5)	26 (6-164) **(p=0.001)***
CASS-positive	142/452 (31)	82/201 (41)	60/249 (24) **(p<0.001)***	56/123 (46)	38/73 (52)	18/48 (38) (p=0.116)*	86/329 (26) **(p<0.001)†**	44/128 (34) **(p=0.014)†**	42/201 (21) **(p=0.007)*** **(p<0.001)†**
CFU	4 (1-11)	3.5 (1-10)	3 (1-9) (p=0.429)*	5 (2-18)	4 (2-16)	2 (1-20) (p=0.757)*	3 (1-7) (p=0.163)†	2 (1-6) (p=0.131)†	3 (1-5) (p=0.523)* (p=0.525)†
1-9 CFU ≥10 CFU	100/142 (70) 42/142 (30)	60/82 (73) 22/82 (27)	40/60 (67) 20/60 (33) (p=0.402)*	34/56 (61) 22/56 (31)	24/38 (63) 14/38 (37)	10/18 (55) 8/18 (45) (p=0.546)*	66/86 (77) 20/86 (23) **(p=0.041)†**	36/44 (82) 8/44 (18) (p=0.057)†	30/42 (71) 12/42 (29) (p=0.254)* (p=0.232)†
Days treatment	4 (2-6) **(p<0.001)‡**	3 (1-5) **(p<0.001)‡**	6 (1-15) (p=0.122)* **(p=0.018)‡**	1 (1-2) (p=0.921)‡	1 (1-2) (p=0.470)‡	1 (1-2) (p=0.068)* (p=0.668)‡	5 (4-13) **(p<0.001)‡**	4 (3-5) (p=0.369)‡	6 (4-20) **(p<0.001)*** **(p=0.024)‡**

*P-values for comparisons between Groups 2 and 3 vs. Group 1 within patients of the same treatment status (i..e, within the all, no treatment or treatment ≤48 h, or treatment 48 h categories)

†P-values for comparions between patients of the same group (Overall, Groups 2 and 3, or Group 1) of different treatment status (no treatment or treatment ≤48 h vs. treatment 48 h)

‡P-values for comparisons within columns for CASS-positives vs. –negatives.

Abbreviations: CASS, cough aerosol sampling system; CFU, colony-forming units; IQR, interquartile range.

Table 5. Time-specific proportion of patients CASS-positive by the number of days on TB treatment prior to the first CASS, stratified by drug susceptibility. Despite being on likely effective treatment for seven days, almost 1/5 (20%) Group 1 patients were CASS-positive at recruitment. Patients who first received treatment on the same day as their baseline CASS were included in the day one row. Data are % (n/N).

Treatment duration (days)	All	Group 1	Groups 2 and 3
1	60 (25/42)	63 (17/27)	53 (8/15) (p=0.542)
2	46 (13/28)	55 (12/22)	17 (1/6) (p=0.099)
3	28 (9/32)	35 (9/26)	0 (0/6) (p=0.089)
4	36 (13/36)	35 (11/31)	40 (2/5) (p=0.845)
5	33 (16/49)	32 (12/37)	33 (4/12) (p=0.954)
6	28 (13/46)	27 (7/26)	30 (6/20) (p=0.818)
7	0 (0/4)	- (0/0)	0 (0/4)
8	43 (3/7)	63 (17/27)	25 (1/4) (p=0.151)
≥14	16 (21/132)	0 (0/1)	16 (21/131)

92 **Table 6.** Individual patient regimens according to drug susceptibility profile (patient group).

	Group 1 (n=198)	**Group 2** (n=129)	**Group 3** (n=95)
First-line*	**195**	**4**	**5**
RIF/INH/PZA/EMB	195	4	5
FQ-based plus AMI or KAN	**2**	**58**	**11**
INH/MOX/KAN/PZA/EMB/ETH/TERI	0	17	3
MOX/KAN/PZA/ETH/TERI	0	7	0
INH/MOX/KAN/PZA/EMB/TERI	0	6	0
MOX/KAN/PZA/EMB/ETH/TERI	1	2	1
INH/MOX/KAN/PZA/ETH/TERI	0	4	0
INH/OFL/KAN/PZA/ETH/TERI	1	1	1
INH/MOX/KAN/PZA/EMB/ETH/TERI/PAS	0	1	1
INH/MOX/KAN/PZA/EMB/ETH/TERI/PAS/CLF	0	1	1
OFL/KAN/PZA/ETH/TERI	0	2	0
INH/MOX/KAN/EMB/ETH/TERI	0	2	0
INH/OFL/KAN/PZA/EMB/ETH/TERI	0	2	0
INH/MOX/KAN/PZA/EMB/ETH	0	2	0
MOX/AMI/PZA/EMB/TERI/PAS/CLF/CAP	0	1	0
MOX/KAN/PZA/EMB/ETH/TERI/PAS	0	1	0
INH/MOX/KAN/PZA/EMB/ETH/TERI/PAS/CLF/CAP	0	0	1
INH/MOX/KAN/PZA/EMB/ETH/CLF	0	1	0
INH/MOX/KAN/PZA/EMB/ETH/TERI/CLF	0	0	1
MOX/KAN/PZA/EMB/ETH/TERI/PAS/CLF	0	1	0
INH/MOX/KAN/ETH/PAS	0	1	0
INH/MOX/KAN/PZA/ETH/TERI/PAS/CLF	0	0	1
MOX/OFL/KAN/PZA/EMB/ETH/TERI/PAS/CLF	0	1	0
MOX/KAN/PZA/EMB/ETH/TERI/CAP	0	1	0
OFL/KAN/PZA/EMB/ETH/TERI	0	1	0
INH/OFL/KAN/PZA/TERI	0	1	0
INH/MOX/KAN/PZA/TERI/CLF	0	0	1
INH/MOX/KAN/TERI	0	1	0
MOX/KAN/PZA/TERI	0	1	0
FQ-based plus CAP	**0**	**43**	**56**
INH/MOX/PZA/EMB/ETH/TERI/PAS/CLF/CAP	0	16	19
INH/MOX/PZA/EMB/TERI/PAS/CLF/CAP	0	6	3
MOX/PZA/EMB/ETH/TERI/PAS/CLF/CAP	0	2	6
MOX/PZA/EMB/TERI/PAS/CLF/CAP	0	0	6
INH/MOX/EMB/TERI/PAS/CLF/CAP	0	3	1
INH/MOX/PZA/EMB/ETH/TERI/PAS/CAP	0	2	2
INH/MOX/PZA/ETH/TERI/PAS/CLF/CAP	0	1	3
INH/MOX/EMB/ETH/TERI/PAS/CLF/CAP	0	2	1
MOX/PZA/ETH/TERI/PAS/CLF/CAP	0	2	1
MOX/EMB/TERI/PAS/CLF/CAP	0	1	2
INH/MOX/PZA/EMB/ETH/TERI/CLF/CAP	0	0	2
MOX/PZA/EMB/ETH/TERI/PAS/CAP	0	1	1
MOX/PZA/EMB/PAS/CLF/CAP	0	0	2
MOX/PZA/EMB/ETH/TERI/CLF/CAP	0	0	2
INH/MOX/PZA/EMB/ETH/TERI/PAS/CAP	0	1	0
INH/MOX/PZA/EMB/ETH/TERI/PAS/CLF/CAP	0	1	0
INH/MOX/ETH/TERI/PAS/CLF/CAP	0	0	1
INH/MOX/PZA/EMB/PAS/CLF/CAP	0	1	0
INH/MOX/PZA/EMB/TERI/CLF/CAP	0	0	1
INH/MOX/PZA/ETH/TERI/CLF/CAP	0	1	0
INH/MOX/PZA/EMB/ETH/CLF/CAP	0	1	0
INH/MOX/PZA/EMB/ETH/PAS/CAP	0	0	1

MOX/PZA/ETH/TERI/CAP	0	0	1
MOX/PZA/EMB/TERI/CLF/CAP	0	1	0
INH/MOX/OFL/PZA/EMB/ETH/TERI/PAS/CAP	0	0	1
MOX/TERI/PAS/CLF/CAP	0	1	0
SLID-based (no FQ)	**0**	**9**	**5**
KAN-based	**0**	**5**	**0**
INH/KAN/PZA/ETH/TERI/PAS	0	1	0
INH/KAN/PZA/ETH/TERI	0	1	0
KAN/PZA/EMB/ETH	0	1	0
INH/KAN/PZA/EMB/ETH/TERI	0	1	0
INH/KAN/PZA/TERI/CLF	0	1	0
CAP-based	**0**	**4**	**5**
INH/PZA/EMB/ETH/TERI/PAS/CLF/CAP	0	2	1
PZA/EMB/TERI/PAS/CLF/CAP	0	1	1
INH/PZA/EMB/ETH/PAS/CLF/CAP	0	1	0
PZA/TERI/PAS/CLF/CAP	0	0	1
INH/PZA/EMB/ETH/TERI/PAS/CAP	0	0	1
PZA/EMB/TERI/PAS/CAP	0	0	1
CLF-based (no FQ, no SLID)			
PZA/TERI/PAS/CLF	0	0	3
PZA/TERI/PAS/CLF	0	0	2
INH/PZA/EMB/ETH/TERI/PAS/CLF	0	0	1
INH/PZA/TERI/PAS/CLF	0	1	0
PZA/EMB/TERI/PAS/CLF	0	0	1
INH/PZA/TERI/CLF	0	0	1
INH/PZA/TERI/PAS/CLF	0	0	1
PZA/TERI/PAS/CLF	0	0	3

[*]Includes one Group 1 patient with ETH substituted for INH

Abbreviations: RIF, rifampicin; INH, isoniazid; PZA, pyrazinamide; EMB, ethambutol; MOX, moxifloxacin; OFL, ofloxacin; AMI, amikacin; KAN, kanamycin; CAP, capreomycin; ETH, ethionamide; PAS, p-aminosalicylic acid; CLF, clofazimine; LNZ, linezolid; SLID, second line injectable drug; TERI, terizidone; CLA, clarithromycin.

Table 7. Inter-isolate single nucleotide polymorphism (SNP) comparisons of 326 isolates by cough aerosol sampling (CASS) status. All SNPs identified were used, and low coverage positions (<10x depth) were excluded on a per pair basis. Edge directionality was determined using patients' date of diagnosis. Differences between the number of linked isolates by CASS status and the number of connections to and from isolates were tested using Fisher's Exact test. The table below summarises the results with varying SNP distances between CASS-positive and CASS-negative patients. An interactive version is available at: https://semiquant.shinyapps.io/CASS_Interactions.

	Clustered Isolates		Connections (edges) between isolates (degree)		Connections (edges) between isolates with direction considered		CASS negative (n=208)		CASS positive (n=118)	
Inter-isolate SNP threshold	Odds Ratio (95% CI)	p value	Odds Ratio (95% CI)	p value	Odds Ratio (95% CI)	p value	n in cluster	Median number of connections (IQR)	n in cluster	Median number of connections (IQR)
10	1.06 (0.65, 1.74)	0.905	0.73 (0.44, 1.21)	0.230	0.81 (0.40, 80)	0.618	80	1.5 (1, 2)	37	1.5 (1, 2)
9	1.07 (0.61, 1.89)	0.893	0.67 (0.40, 1.13)	0.140	0.71 (0.32, 74)	0.458	74	1.5 (1, 2)	32	1.5 (1, 2)
8	0.82 (0.42, 1.59)	0.638	0.80 (0.46, 1.37)	0.443	0.71 (0.27, 62)	0.505	62	1.5 (1, 2)	30	1.5 (1, 2)
7	0.77 (0.36, 1.66)	0.588	0.83 (0.46, 1.47)	0.586	0.66 (0.21, 51)	0.446	51	1.5 (1, 2)	25	1.5 (1, 2)
6	0.63 (0.26, 1.46)	0.322	0.91 (0.48, 1.69)	0.883	0.69 (0.19, 40)	0.566	40	1.5 (1, 2)	21	1.4 (1, 2)
5	0.61 (0.23, 1.60)	0.375	0.99 (0.50, 1.93)	1	0.84 (0.19, 32)	1	32	1.6 (1, 2)	18	1.4 (1, 2)
4	0.42 (0.10, 1.57)	0.241	0.79 (0.33, 1.75)	0.582	0.82 (0.13, 24)	1	24	1.6 (1, 2)	11	1.4 (1, 2)
3	0.32 (0.04, 1.83)	0.259	1.04 (0.41, 2.51)	1	0.47 (0.02, 17)	0.584	17	1.6 (1, 2)	10	1.3 (1, 2)
2	0.21 (0.02, 1.72)	0.193	1.19 (0.41, 3.27)	0.811	0.60 (0.02, 12)	1	12	1.6 (1, 2)	8	1.3 (1, 1)
1	0 (0.00, 5.05)	0.429	0.58 (0.06, 3.32)	0.716	0 (0.00, 6)	1	6	1.7 (1, 2)	2	1.0 (1, 1)
0	0 (0.00, 39.00)	1	1.77 (0.02, 139.39)	1	0 (0.00, 1)	1	1	2.0 (2, 2)	1	1.0 (1, 1)

Table 8. Univariate and multivariate logistic regression analyses of drug-related predictors of CASS-positivity in sputum culture-positive patients on different treatment regimens. Each model (all patients, first-line regimen, FQ-regimen) was individually adjusted using the non-drug predictors of cough aerosol culture-positivity in sputum culture-positive patients shown in **Supplementary Table 8**. Data are median (IQR) or n/N (%) unless otherwise stated.

	Univariate analyses				**Multivariate analyses†**	
	All patients on treatment					
	CASS-negative	**CASS-positive**	**Unadjusted odds ratio** (95% CI)	**P-value**	**Adjusted odds ratio** (95% CI) (n=269)	**P-value**
Days treatment	6 (4-56)	4 (2-6)	0.99 (0.99, 1.00)	0.065	-	-
No. drugs	6 (4-7)	4 (4-6)	0.75 (0.65, 0.87)	**<0.001**	-	-
Regimen						
First-line	91/163 (56)	72/163 (44)	Reference	-	Reference	-
FQ-based	118/148 (80)	30/148 (20)	0.32 (0.19, 0.53)	**<0.001**	0.34 (0.17, 0.65)	**0.001**
SLID- or CLF-based (no FQ)	8/13 (62)	5/13 (39)	0.79 (0.25, 2.52)	0.690	2.17 (0.34, 13.96)	0.413
	First-line regimen					
	CASS-negative	**CASS-positive**	**Unadjusted odds ratio** (95% CI)	**P-value**	**Adjusted odds ratio** (95% CI) (n=145)	**P-value**
Days treatment	4 (2-5)	3 (1-5)	0.88 (0.74, 1.04)	0.132	No drug-related variables significant in univariate analyses. None included in multivariate analysis.	
No. likely effective drugs	4 (3.5-4)	4 (3-4)	0.96 (0.61, 1.52)	0.868		
Drug likely effective						
RIF	77/83 (93)	64/67 (95)	0.84 (0.47, 1.51)	0.570		
INH	75/92 (91)	57/67 (85)	0.53 (0.20, 1.41)	0.203		
PZA	70/72 (97)	54/57 (95)	1.00 (0.90, 1.12)	0.948		
EMB	82/83 (99)	67/67 (100)	0.74 (0.28, 1.92)	0.534		
	FQ-based regimen					
	CASS-negative	**CASS-positive**	**Unadjusted odds ratio** (95% CI)	**P-value**	**Adjusted odds ratio** (95% CI) (n=141)	**P-value**
Days treatment	39 (6-168)	48 (6-166)	1.00 (0.99, 1.00)	0.894	-	-
No. likely effective drugs	3 (2-4)	3 (2-4)	0.78 (0.58, 1.04)	**0.088**	0.68 (0.41, 1.14)	0.151
Drug likely effective						
FQ	44/111 (40)	5/27 (19)	0.38 (0.15, 1.02)	**0.057**	0.99 (0.25, 3.84)	0.992
SLID	50/98 (51)	12/24 (50)	0.96 (0.38, 2.42)	0.931	-	-
PZA	29/104 (36)	7/23 (30)	0.87 (0.73, 1.04)	0.129	-	-

EMB	29/86 (34)	7/23 (30)	0.75 (0.39, 1.46)	0.407	-	-
ETH	23/90 (26)	1/23 (4)	0.13 (0.20, 1.04)	**0.054**	0.16 (0.02, 1.41)	**0.099**
PAS	57/63 (91)	14/17 (82)	0.49 (0.11, 2.21)	0.354	-	-
CLZ	51/51 (100)	17/17 (100)	Non-calculable	-	Not included as no resistance observed	-

[†]Values as in the final model.

For second-line regimens not comprised of a FQ, analyses were not done due to the small number of CASS-negative (n=9) and CASS-positive (n=5) patients.

Abbreviations: CASS, cough aerosol sampling system; CI, confidence interval; IQR, interquartile range.

Table 9. Non-drug predictors of cough aerosol culture-positivity in sputum culture-positive patients on treatment used to adjust models in **Supplementary Table 7.** Data are median (IQR) or n/N (%) unless otherwise stated.

	Univariate analyses				**Multivariate analysis†**	
	All patients on treatment					
	CASS-negative	**CASS-positive**	**Unadjusted odds ratio** (95% CI)	**P-value**	**Adjusted odds ratio** (95% CI) (n=269)	**P-value**
Clinical characteristics						
TB symptom score	4 (2-5)	3 (2-4)	0.88 (0.78, 0.98) (per unit)	**0.017**	0.80 (0.68, 0.94) (per unit)	**0.007**
HIV-positive	87/210 (41)	30/106 (28)	0.47 (0.29, 0.78)	**0.003**	0.44 (0.22, 0.86)	**0.017**
PCF (l/min)	293 (240-363)	337 (286-400)	10.06 (10.03-10.09) (per 10 units)	**<0.001**	10.08 (10.04, 10.13) (per 10 units)	**<0.001**
Sputum microbiology						
Culture TTP (days)	12 (8-16)	8 (6-10)	0.80 (0.75-0.86)	**<0.001**	0.75 (0.68, 0.83) (per unit)	**<0.001**
	First-line regimen					
	CASS-negative	**CASS-positive**	**Unadjusted odds ratio** (95% CI)	**P-value**	**Adjusted odds ratio** (95% CI) (n=145)	**P-value**
Clinical characteristics						
TB symptom score	4 (3-5)	3 (2-4)	0.74 (0.61, 0.90) (per unit)	**0.003**	0.75 (0.59, 0.95) (per unit)	**0.019**
HIV-positive	42/83 (51)	21/69 (30)	0.43 (0.22, 0.83)	**0.013**	0.30 (0.13, 0.70)	**0.005**
PCF (l/min)	307 (250-353)	345 (290-417)	10.08 (10.04-10.13) (per 10 units)	**<0.001**	10.11 (10.04, 10.16) (per 10 units)	**<0.001**
Sputum microbiology						
Culture TTP (days	11.5 (8-15)	9 (7-10)	0.86 (0.79-0.93)	**<0.001**	0.80 (0.71, 0.90) (per unit)	**<0.001**
	FQ-based regimen					
	CASS-negative	**CASS-positive**	**Unadjusted odds ratio** (95% CI)	**P-value**	**Adjusted odds ratio** (95% CI) (n=141)	**P-value**
Clinical characteristics						
TB symptom score	4 (2-5)	3 (1-4)	0.92 (0.78, 1.09) (per unit)	0.357	-	-
HIV-positive	41/114 (36)	7/29 (24)	0.57 (0.22, 1.44)	0.232	-	-
PCF (l/min)	283 (230-363)	340 (268-393)	10.04 (9.99-10.01) (per 10 units)	0.138	-	-
Sputum microbiology						
Culture TTP (days	13 (9-17)	7 (5-8)	0.71 (0.61-0.83)	**<0.001**	0.75 (0.62, 0.87) (per unit)	**<0.001**

†Values as in the final model.

Abbreviations: CASS, cough aerosol sampling system; CI, confidence interval; IQR, interquartile range; TTP, time-to-positivity; MDR-, multidrug-resistant; PCF, peak cough flow.

Table 10. List of cough aerosol sampling system (CASS), sputum smear microscopy, sputum Xpert MTB/RIF, and sputum MGIT960 liquid culture reproducibility results in pre-treatment patients newly programmatically diagnosed as Xpert MTB/RIF-positive.

	Day 1								Day 2							
	Morning				Afternoon				Morning				Afternoon			
Patient	CASS CFU	Smear grade	Xpert (semi-quantitation level, C_{Tmin})	Culture (TTP)	CASS CFU	Smear grade	Xpert (semi-quantitation level, C_{Tmin})	Culture (TTP)	CASS CFU	Smear grade	Xpert (semi-quantitation level, C_{Tmin})	Culture (TTP)	CASS CFU	Smear grade	Xpert (semi-quantitation level, C_{Tmin})	Culture (TTP)
R1	0	N	P (M, 20.3)	P (13)	0	P+	P (M, 21.1)	N	0	P (SC)	P (M, 21.6)	N	0	N	P (L, 22.3)	N (0)
R2	15	P+++	P (H, 11.4)	P (4)	7	P+++	P (H, 13.2)	P (4)	27	P+++	P (M, 19.7)	P (5)	0	P+++	P (M, 17.2)	P (4)
R3	1	N	P (H, 15.6)	P (7)	2	P+	P (H, 16.3)	P (8)	0	P+++	P (M, 20.1)	P (6)	0	P+	P (M, 18.7)	P (6)
R4	51	P+++	P (H, 14.5)	P (4)	48	P+++	P (H, 14.4)	P (3)	20	P+++	P (H, 14.2)	P (5)	33	P++++	P (H, 15.4)	P (3)
R5	0	P+++	P (L, 24.3)	P (6)	0	P+	P (M, 18.3)	P+C†	0	P+++	P (H, 16.1)	P (6)	0	P+++	P (M, 21.5)	P (7)
R6	184	P++	P (M, 18.2)	P (4)	4	P+	P (H, 15.7)	P (5)	124	P+	E (0)	P (5)	26	P+++	P (M, 16.5)	P (5)
R7	9	P+++	P (M, 17.9)	P+C†	0	P+++	P (M, 19.4)	P (6)	4	P+++	P (H, 15.5)	P (6)	4	P++	P (H, 15.4)	P (6)
R8	1	N	P (M, 21.7)	P (11)	1	N	P (L, 22.4)	P (10)	0	P (SC)	P (L, 22.7)	P (13)	0	P (SC)	P (L, 22.3)	P (11)
R9	2	P+++	P (H, 16.3)	P (4)	2	P+++	P (H, 14.6)	P (4)	2	P+++	P (H, 14.9)	P (5)	3	P+++	P (H, 15.2)	P (4)
R10	0	N	N	N	0	N	N	C†	0	N	N	C†	0	N	N	N
R11	1	P+	P (M, 19.3)	P (9)	9	P++	P (M, 17.2)	P (8)	5	P++	P (M, 18.6)	P (8)	1	P (SC)	P (M, 17.1)	P (10)
R12	C	P+++	P (H, 11.3)	P (3)	191	P+++	P (H, 15.8)	P (3)	21	P++	P (H, 15.4)	P (5)	20	P++	P (H, 11.6)	P+C†
R13	C	N	P (M, 18.8)	N	4	P+++	P (H, 15.5)	P (6)	3	P++	P (M, 19.4)	P (9)	8	P++	P (M, 17.8)	P (6)
R14	11	P+	P (H, 15)	P (6)	12	P+	P (M, 16.5)	P (4)	0	P+++	P (H, 13.5)	P (6)	4	P+++	P (H, 13.4)	P (3)
R15	3	P+++	P (H, 15.4)	P (6)	13	P+++	Not done*	P (5)	14	P++	P (H, 15.1)	P (6)	6	P+++	P (H, 14.6)	P (7)
R16	16	P++	P (M, 18.4)	P (4)	0	P+++	P (H, 16.1)	P (3)	21	P+++	P (M, 16.5)	P (6)	45	P+++	P (H, 16.1)	P (6)
R17	9	P++	P (H, 16)	P (4)	7	P++	P (M, 17.3)	P (3)	0	P+++	P (H, 13.4)	P (3)	9	P+++	P (H, 14.4)	P (3)
R18	13	P++	P (H, 12.9)	P (8)	12	P++	P (M, 17.1)	P (9)	4	P++	P (H, 14.6)	P (9)	0	N/A	P (M, 17)	P (18)
R19	25	N	P (M, 21.2)	P (10)	3	N	P (M, 20.4)	P (9)	17	P+	P (M, 21.5)	P (10)	0	N	P (L, 22.8)	P (10)
R20	0	P++	P (M, 17.6)	P (7)	20	P+++	P (M, 17.4)	P (5)	0	P (SC)	P (H, 15.1)	P (8)	12	P++	P (H, 14.6)	P (6)
R21	34	P+	P (M, 16.5)	P (8)	43	P+++	P (H, 16.2)	P (9)	18	P++	P (M, 17.8)	P (5)	28	P+++	P (M, 19.6)	P (5)
R22	0	P++	P (M, 17.2)	P (9)	5	P++	P (M, 16.7)	P (7)	0	P+++	P (H, 15.4)	P (9)	2	P++	P (M, 22.1)	P (8)
R23	2	P++	P (M, 19.3)	P (11)	2	P++	P (M, 19.3)	P (11)	0	P++	P (L, 23.7)	P (9)	1	P++	P (M, 19.8)	P (8)
R24	284	P++++	Not done*	P (8)	9	P+++	P (M, 17.5)	P (6)	86	P+++	P (M, 17.1)	P (9)	6	P+++	P (H, 15.5)	P (7)
R25	0	P+++	P (M, 17)	P+C†	0	P+++	P (M, 19.9)	P (5)	0	P+++	Not done*	P (5)	C	Not done*	Not done*	Not done*
R26	4	P++	P (M, 17.4)	N	1	P+	P (M, 16.5)	P (5)	0	P+++	P (H, 15.2)	P (6)	0	P+	P (M, 17.6)	P (8)
R27	0	P++++	P (H, 11.4)	P (2)	52	P+++	P (H, 12.4)	P (3)	0	P++++	P (H, 10.5)	P (3)	0	P+++	Not done*	P (3)
R28	41	P+++	P (H, 12.3)	P (3)	0	P++	P (H, 14.8)	P (5)	0	P++	P (H, 12.7)	P (3)	0	P+	P (H, 13.2)	P (5)
R29	0	P+++	P (H, 18.2)	P (5)	0	P++	P (H, 19.2)	P (8)	0	P+	P (H, 18.4)	P (9)	0	P+	P (H, 21.6)	P (9)
Proportion positive (%)	19/27 (70)	23/29 (79)	27/28 (96)	26/29 (90)	21/29 (72)	26/29 (90)	27/28 (96)	27/28 (96)	14/29 (48)	28/29 (97)	26/27 (96)	27/28 (96)	16/28 (58)	24/28 (86)	26/27 (96)	26/28 (93)

*Missing data: Xpert (n=3), if patients could produce one sputum it was used for microscopy and culture; CASS (n=1), patient unwell and CASS not done; smear microscopy and culture (n=1), patient unable to produce sputum. †TTP not given if contamination was observed. Abbreviations: P, positive; N, negative; C, contamination; CFU, colony forming units; C_{Tmin}, minimum cycle threshold value; E, error; H, high; M, medium; L, low; VL, very low; SC, scanty; TTP, time-to-positivity.

Table 11. Cough aerosol sampling and sputum microbiology reproducibility. CASS grade was classified based on the number of CFU in aerosol (no growth = 0, 1-9 CFU = 1, ≥10 CFU = 2). Data are shown with 95% CI.

	Overall	Day 1 Morning vs. Day 1 Afternoon	Day 2 Morning vs. Day 2 Afternoon	Day 1 Morning vs. Day 2 Morning	Day 1 Afternoon vs. Day 2 Afternoon
Smear status, kappa	0.46 (0.13, 0.84)	0.61 (0.23, 1.00)	0.36 (-0.16, 0.89)	0.24 (-0.15, 0.64)	0.51 (0.03, 0.99)
Smear grade, kappa	0.23 (0.14, 0.30)	0.34 (0.26, 0.47)	0.27 (0.24, 0.27)	0.12 (-0.02, 0.23)	0.26 (0.14, 0.28)
Culture status, kappa	0.54 (0.37, 1.00)	-0.05 (-0.15, 0.05)	1.00 (1.00, 1.00)	-0.05 (-0.15, 0.05)	1.00 (1.00, 1.00)
TTP, average ICC	0.91 (0.83, 0.96)	0.93 (0.82, 0.97)	0.80 (0.55, 0.91)	0.91 (0.79, 0.96)	0.80 (0.53-0.91)
Xpert status, kappa	1.00 (1.00, 1.00)	1.00 (1.00, 1.00)	1.00 (1.00, 1.00)	1.00 (1.00, 1.00)	1.00 (1.00, 1.00)
Xpert grade, kappa	0.33 (0.15, 0.47)	0.41 (0.20, 0.51)	0.43 (0.29, 0.48)	0.41 (0.27, 0.64)	0.24 (0.16, 0.56)
C_{Tmin}, average ICC	0.86 (0.74, 0.94)	0.82 (0.58, 0.92)	0.82 (0.59, 0.92)	0.72 (0.37, 0.88)	0.73 (0.389, 0.88)
CASS status, kappa	0.37 (0.29, 0.56)	0.47 (0.10, 0.83)	0.43 (0.10, 0.76)	0.50 (0.23, 0.78)	0.31 (-0.03, 0.65)
CASS grade, kappa	0.38 (0.22, 0.56)	0.39 (0.09, 0.43)	0.45 (0.29, 0.66)	0.51 (0.40, 0.62)	0.36 (0.26, 0.56)
CASS CFU, average ICC	0.59 (0.25, 0.80)	0.04 (-1.10, 0.56)	0.46 (-1.68, 0.75)	0.80 (0.56, 0.91)	0.32 (-0.47, 0.69)

Kappa interpretation: 0.01-0.20 slight; 0.21-0.40 fair; 0.41-0.60 moderate; 0.61-0.80 substantial; 0.81-1.00 almost perfect.[3]

ICC reliability interpretation: <0.5 poor, 0.5-0.75 moderate, 0.75-0.90 good, >0.90 almost perfect.[4]

Abbreviations: CASS, cough aerosol sampling system; CI, confidence interval; CFU, colony forming units; C_{Tmin}, minimum cycle threshold value; ICC, intraclass correlation; TTP, time-to-positivity; Xpert, Xpert MTB/RIF.

Supplementary Results

Sputum Auramine-Nile Red staining

Initially, Auramine-Nile Red straining was done on all Ziehl-Neelson smear-positive patients; however, due to low numbers of bacilli observed after Auramine-Nile Red straining - even after extensive optimisation and troubleshooting - we changed our protocol to visualise slides only from patients who had a Ziehl-Neelson sputum smear-grade of two or higher.

Of the 194 sputum smear grade 2+ or greater patients, 66 (34%) had at least one Auramine-positive bacilli after Auramine-Nile Red straining and all but 3/66 (5%) had Nile-Red co-localization. Overall, the mean (standard deviation) proportion of Auramine-positives bacilli co-localization was 0.76 (0.47). In CASS-negative vs. positive-patients, no differences were detected [0.75 (0.30) vs. 0.74 (0.30); p=0.644].

Genome wide association studies

We investigated if nonsynonymous SNPs on a gene level in *M. tuberculosis* from sputum culture isolates were associated with CASS-positivity. This analysis included 318 isolates with 115 (36%) from CASS-positive individuals and 857 genes. We detected no associations with CASS-status in univariate, logic regression (interaction analysis), phylogenetic convergence, or efficient mix-models analyses (Figure 3). We also did not detect associations using a multivariate logistic model that included two principal components, patient age, TB symptom score, HIV-status, duration of treatment less than 48h, PCF, culture time to positivity, and drug-resistance category. An efficient mixed model analysis using 274 isolates (101 from CASS-positive) for sputum culture time to positivity did not detect significant associations (Figure S3). We further investigated the associations of structural variants with CASS-positivity using 318 isolates with 115 (36%) from CASS-positive individuals and 73 indels. We detected no

153 indels associated with CASS-status in logic regression, phylogenetic convergence, or efficient
154 mix-models analyses.

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Appendix IV

Direct genotyping of Mycobacterium tuberculosis from Xpert® MTB/RIF remnants.

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Direct genotyping of *Mycobacterium tuberculosis* from Xpert® MTB/RIF remnants

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ABSTRACT

Genotyping of *Mycobacterium tuberculosis* (MTB) isolates has markedly improved our knowledge of its transmission dynamics. MIRU-VNTR is considered the reference molecular tool for MTB fingerprinting. However, the dependence of this technique on cultured isolates means that we lack molecular epidemiology data from many settings where culture facilities have not been implemented. Efforts have been made to adapt the MIRU-VNTR procedure to direct analysis of clinical specimens, although implementation of these efforts has not proven successful. The large-scale roll-out of Xpert MTB/RIF (Xpert) technology, which is now in almost every TB-endemic country, including many where MTB is not cultured, provides us with a new opportunity to explore whether MTB genotyping could be performed from the remnants of the Xpert cartridge. We ran a pilot study in Mozambique in which the remnants of 24 positive Xpert assays for detection of MTB were used as template material for the 15-locus or the more discriminatory 24-locus MIRU-VNTR technique. MTB fingerprinting was possible in specimens with a high bacterial burden, according to the Xpert load categories, and within the first week after Xpert was performed. Given the wide availability, simple processing, and rapid reporting of results with Xpert, our findings suggest that MIRU-VNTR–based fingerprinting from remnants of Xpert could play a major role in extending MTB molecular epidemiology studies to settings where information on the transmission dynamics of this pathogen is lacking.

1. Introduction

Molecular epidemiology studies help us to identify which patients within a population are in clusters, thus indicating that they belong to the same transmission chain, useful indicators to evaluate the efficiency of control programs.

The MIRU-VNTR technique optimized MTB fingerprinting for epidemiological purposes. It analyses the number of tandem repeats from 15 loci or, in its most discriminatory format, 24 loci [1]. MIRU-VNTR still needs to be applied on cultured isolates to ensure complete typing. Consequently, there are knowledge gaps in the molecular epidemiology of TB in many low- and middle-income countries, where MTB culture facilities are not widely available.

Efforts have been made to adapt MIRU-VNTR to enable direct analysis of the bacilli present in respiratory specimens [2]. This approach opens the door to new culture-independent genotyping strategies. However, these new developments still require specific laboratory facilities for basic sample processing, decontamination, and inactivation of sputa, thus explaining why they have not been extensively applied.

The new, rapid, automated test Xpert® MTB/RIF (Cepheid, Sunnyvale, USA) (Xpert) was developed for detection of both MTB and rifampin resistance, directly from respiratory samples [3]. This assay needs no laboratory infrastructure, as the sputa can be mixed directly

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with an inactivation and liquefaction reagent before being transferred into a cartridge. Since the GeneXpert system is distributed worldwide, it provides us with an excellent opportunity to evaluate if it is possible to perform culture-independent MTB genotyping directly from the cartridge remnants.

Some studies have shown the feasibility of using Xpert remnants, i.e. leftover diluted samples, or cartridge extracts, to perform genotypic drug susceptibility testing, spoligotyping [4,5] or Allele-Specific-Oligonucleotide PCR [6]. Additionally, only one single effort of characterization by VNTR analysis has been made on Xpert remnants, from leftover diluted samples, and just limited to the 4 VNTR loci set recommended for Beijing strains genotyping, with non-optimal results [5]. Here, we present the results of the first pilot study to evaluate the feasibility of performing standard MIRU-VNTR analysis from the remnants from the sample chamber of the Xpert cartridge.

2. Materials and methods

2.1. Study setting

The study was performed at the Manhiça Health Research Center (CISM), located in Manhiça, a low-resource, rural area with a high TB and HIV burden in Southern Mozambique [7]. Mozambique is one of the 30 World Health Organization's high burden countries [8]. TB has been epidemic in the country, with high mortality, and a low case detection rate [9].

2.2. Ethics

The CISM Internal Scientific Committee and the Mozambican National Bioethics Committee approved the research protocol, which included the present study (Ref 25/CNBS/16). Informed consent was obtained.

2.3. Clinical specimens

Respiratory samples received for Xpert analysis (March 1st to June 3rd, 2016) were decontaminated [10] and mixed in a 1:2 proportion with the Xpert Sample Reagent. Two milliliters were used for the test following the manufacturer's instructions. Once the Xpert run had finished, the remnant volume from the sample chamber of the cartridge was transferred to a tube and stored at 4 °C.

2.4. DNA purification

DNA was purified from the Xpert remnant using a column-based method (QIAamp DNA minikit; Qiagen, Courtaboeuf, France) following the manufacturer's instructions with only minor modifications. The supernatant after the initial centrifugation was also passed through the column to recover any free DNA. The final elution volume in buffer AE varied (85 or 130 µl) depending on the MIRU-VNTR format (15- or 24-locus).

2.5. MIRU-VNTR analysis

MIRU-VNTR was performed based on a simplex-PCR format as described elsewhere [11].

2.5.1. Non-optimized starting protocol

The starting protocol was as follows: 5 µl of purified DNA was added to a final reaction volume of 50 µl containing 0.2 µl of DNA Polymerase (GoTaq® Hot Start Polymerase Kit [Promega]), 10 µl of Colorless GoTaq® Flexi Buffer (1 ×), 0.2 mM of dNTPs, 1.5 or 3 mM of $MgCl_2$ (3 mM for VNTR 580, 802, 960, 2401, 2996, and 3690, and 1.5 mM for the remaining loci) and 0.1 µM of each unlabelled oligonucleotide.

The PCR conditions were 95°C for 15 min followed by 40 cycles of 95°C for 1 min, 54°C (for loci VNTR 154, 580, 820, 960, 1644, 2059, 2531, 2687, 2996, 3007, 3192, and 4348) or 59°C (for the remaining loci) for 1 min, and 72°C for 1 min and 50 s. A final elongation step was run at 72°C for 10 min. PCR products were sized using electrophoresis with 2% agarose gels (mixing NuSieve® 3:1 Agarose [Lonza] with TAE 1 ×).

2.5.2. Optimized protocol

For the loci in which proper amplification was obtained when following the starting protocol, it was considered the final optimized protocol. However, in the case of the VNTR loci 424, 1644, 1955, 2059, 2347, 2531, 2687, 3171, 3192, 4052, 4156, and 4348 the procedure was adjusted to ensure optimal results. The final optimized procedure for these loci consisted of applying the PuReTaq Ready-To-Go PCR beads system (GE Healthcare, Buckinghamshire, United Kingdom). The PCR mixture contained 2.5 µl of DNA, 0.25 µM of each oligonucleotide, and 1.5 µl of DMSO in a final volume of 25 µl.

2.6. Evaluation strategy

Our evaluation strategy was based on a gradient running from less demanding to more demanding circumstances depending on a series of factors: i) the number of MIRU-VNTR loci to be analysed (15 or 24; the need to perform 15 or 24 PCRs determines the final elution volume in the DNA purification procedure and consequently modifies the DNA concentration to be used as the PCR template, which could eventually affect the probability of obtaining appropriate amplification). ii) the bacterial load in the specimens (based on the semiquantitative information provided by the GeneXpert software that distributes the specimens in categories -high, medium, low and very low- depending on the Ct values) and iii) the length of the delay between the Xpert assay and DNA purification-MIRU-VNTR analysis.

In order to estimate a potential association between bacillary burden or delay in MIRU-VNTR analysis with the identification of complete 15 and 24 loci MIRU-VNTR patterns, we compared the number of samples with complete MIRUtypes per bacillary load category and per length of delay (less than 8 vs 8 or more days). We also estimated the mean individual loci identified per category of interest. Given the limited sample size, p-values were obtained using Fisher's exact test.

3. Results and discussion

A total of 27 samples from 26 patients were Xpert-positive, rifampin-susceptible, during the 3-month study period. Only two specimens were monoresistant to INH. The distribution of samples according to bacterial load, based on the interpretation that the GeneXpert software made from the Ct values was: 17, 5 and 5 samples with high, medium and low bacterial loads, respectively. These three semiquantitative categories have been shown to correlate with the bacterial loads determined from acid-fast smears (≥2+, ≥1 + and ≤1+, respectively) [12].

Our first objective was to obtain 15-locus MIRU-VNTR data from the Xpert remnants. We analysed 5 samples with a high bacterial load according to GeneXpert and with a maximum delay of 7 days between Xpert and MIRU-VNTR.

We obtained an interpretable result for 10 of 15 loci in the 5 samples analysed, indicating that further optimization was needed to ensure a full 15-locus MIRU-VNTR analysis. Increasing the primer concentration to 0.4 mM for locus 424 and applying a PCR bead system for the VNTR loci 1644, 1955, 4156, and 4052 led to complete 15-locus MIRU-VNTR patterns in all the specimens analysed (Table 1).

15-locus MIRU-VNTR offers sufficient discriminatory power for epidemiological purposes [1]. However, we also evaluated whether the highest discriminatory format (i.e., 24 loci) could also be obtained from the Xpert remnants when our optimized procedure was applied. In the

Table 1
Results for 15-locus MIRU-VNTR.

Sample	Bacterial Load	Days of delay	424	577	580	802	960	1644	1955	2163b	2165	2401	2996	3192	3690	4052	4156
1	HIGH	0	3[a]	4	2	4	4	3	3	2	2	1	5	3	2	8[b]	3[b]
2	HIGH	7	2[a]	2	5	3	4	1[b]	8[b]	3	9	2	2	5	7	2[b]	1[b]
3	HIGH	0	2[a]	2	5	3	4	3[a]	11	3	9	2	2	5	7	3[b]	1[b]
4	HIGH	2	2[a]	4	5	2	4	3[a]	7	10	7	2	2	5	6	6[b]	1[b]
5	HIGH	6	2	4	2	1	3	3[a]	2	5	3	2	5	3	5	4[b]	2[b]

[a] Application of a final concentration of 0.4 μM for each oligonucleotide.
[b] Application of the PuReTaq Ready-To-Go PCR Beads system.

Table 2
Results for 24-locus MIRU-VNTR.

Sample	Bacterial Load	Days of delay	24-locus MIRU-VNTR																							
			15-locus MIRU-VNTR																							
6	HIGH	4	3[a]	4	2	2	4	3[a]	2	4	2	2	3	3	1	7[b]	2[b]	2	2[b]	4[b]	2	6	1	3	1	2
7	HIGH	4	4[a]	4	2	3	3	3[a]	5	4	4	4	7	5	3	8[b]	2[b]	2	2[b]	4[b]	2	5	1[b]	3	3[b]	2
8	HIGH	7	2[a]	4	5	3	4	3[b]	6	7	8	2	2	4	5	7[b]	1[b]	2	1[b]	3[b]	5	6	2[b]	3	3[b]	7[b]
9	HIGH	1	4[a]	4	6	5	5	7[b]	4	4	10	4	4	5[b]	11	3[b]	1[b]	2	1[b]	3[b]	4	11[b]	8[b]	3	3[b]	1

The order of MIRU-VNTR loci in the Table is: 424, 577, 580, 802, 960, 1644, 1955, 2163b, 2165, 2401, 2996, 3192, 3690, 4052, 4156, 154, 2059, 2347, 2461, 2531, 2687, 3007, 3171, 4348.
[a] Application of a final concentration of 0.4 μM for each oligonucleotide.
[b] Application of the PuReTaq Ready-To-Go PCR Beads system.

second evaluation step, we selected another 4 specimens, again with a high bacterial load and a short delay between Xpert and VNTR analysis (range 1–7 days). Complete 24-locus MIRU-VNTR types (Table 2) were obtained for all the specimens. For 6 of the new 9 loci, the PuReTaq Ready-To-Go PCR Beads system was required (Table 2). We also needed to add the VNTR 3192 locus to those in the 15-locus set that took advantage of the beads system, which thus defined the protocol to be used in the evaluation from this point onward.

We then introduced the impact of the bacterial burden in the specimens in our analysis, by including samples with a lower-than-high load of bacilli, still assuring a maximum delay of 7 days between Xpert and MIRU-VNTR. 4 samples with a medium bacterial load and 3 with a low bacterial load were available for study. We obtained a complete 15-locus MIRU-VNTR pattern in 1 sample with medium bacillary burden and in none with low (Table 3). Considering the complete 15-locus MIRU-VNTR pattern that had been obtained previously from all the high bacterial load specimens, a statistically significant association between bacillary load and ability to obtain a complete 15-loci MIRU type was found (Fisher's exact test p-value: 0.01). The average number of loci with a result per high, medium and low bacillary burden specimens was 15, 13 and 7 respectively.

It is known that the efficiency of amplification is not equivalent for all MIRU loci and it could explain the amplification failures for certain loci when the amount of DNA was expected to be low/very low. In addition, some of the amplification failures could be due to poor DNA quality as result of suboptimal storage conditions of the remnants until analysis: at 4°C, but not frozen, and not neutralizing the pH, a caution introduced in other studies [5]. Only 1 MIRUtype was complete (medium load specimen), and this was possible only thanks to the addition of beads to locus 3690. In the remaining samples, the failed amplifications could not be resolved even by adding beads.

In molecular epidemiology studies, to rule in properly the involvement of an isolate in a cluster, a complete MIRU pattern (at least for the 15-loci) is required. However, for the disease tracing it is also relevant to rule out those cases not related to transmission clusters. For this reason, some of the incomplete patterns obtained from Xpert remnants could still be valuable, as differences at the amplified loci of greater magnitude than the single locus variations tolerated within clusters, would rule out the relationships between the cases.

Newly diagnosed cases will correspond more frequently to cases with a high bacterial load [13,14], due to the diagnostic delay expected in most of the countries where our strategy would improve current procedures. In fact, in our study 63% of the samples recruited corresponded to high bacterial load specimens. This observation would probably minimize the size of the subsample (with lower burden specimens) with incomplete results. However, we must admit that our data indicate that, in absence of culture facilities, a complete fingerprint cannot be always assured for specimens with low/medium bacterial load.

The final step in our evaluation was to assess the impact of a delay in the acquisition of a complete MIRU-VNTR pattern. The rationale behind this decision was the fact that in certain settings, Xpert remnants will have to be transported to other reference laboratories to be genotyped. Until now, we had observed that a delay of up to 1 week does not

Table 3
Results for 15-locus MIRU-VNTR in medium- and low-load samples.

Sample	Bacterial Load	Days of delay	424	577	580	802	960	1644	1955	2163b	2165	2401	2996	3192	3690	4052	4156
10	MEDIUM	5	4	9	7	3	4[a]	–	6	3	9[a]	2[a]	2[a]	5	2	–	1
11	MEDIUM	6	2	4	2	4	3	2	10	3	3	2	5	3	3[a]	4	1
12	MEDIUM	5	2[a]	9	–	3	–	–	12	11[a]	6[a]	2[a]	4	5	2	4	1
13	MEDIUM	4	2	4	–	3	4	3	12	11	6	2	5	5	2	4	1
14	LOW	6	2	4	–	1	–	–	2	–	3	2	–	–	5	–	–
15	LOW	5	–	–	–	5	–	4	5	–	2	8	8	3	6	7	–
16	LOW	5	6	–	–	4	–	–	2	–	–	5	5	1	6	–	–

[a] Application of the PuReTaq Ready-To-Go PCR Beads system.

Table 4
Results of 24-locus MIRU-VNTR for samples processed with longer delays.

Sample	Bacterial Load	Days of delay	24-locus MIRU-VNTR																						
			15-locus MIRU-VNTR																						
17	HIGH	8	1	4	2	1	4	2	3	–	2	1	6	3	2	8	2	2	2	4	2	–	5	2	3	2
18	HIGH	11	2	4	2	1	4	3	2	3	3	4	6	2	2	7	3	2	1	4	3	5	1	3	2	2
19	HIGH	13	2	4	2/5	1/3	3/4	–	12	4	2/9	2	2	3/5	2/6	3	1/2	2	1/2	3/4	1/6	6	1/2	2/3	3	2/3
20	HIGH	13	5[a]	4[a]	2	1	3	1	3	12[a]	2	1[a]	4	3	2	8	2	2	2	3	2[a]	6	5	2	1	2
21	HIGH	15	2	2	5	3	4	3	7	3	9	2	2	4	7	5	1	2	1	3	4	6	–	3	3	1
22	HIGH	23	4	4	2	3	3	3	5	4	4	4	7	5	3	8	2	2	2	4	2	5	1	3	3	2
23	HIGH	28	2	4	2	4	–	–	4	3[a]	2	4	5	2	3	–	3	2	2	–	2	5	1	3	1	2
24	HIGH	31	4	4[a]	2	3	4	–	4	4[a]	2	2	5	3	1	7[a]	2	2	2	4	1	6	1	3	1	2

The order of MIRU-VNTR loci in the Table is: 424, 577, 580, 802, 960, 1644, 1955, 2163b, 2165, 2401, 2996, 3192, 3690, 4052, 4156, 154, 2059, 2347, 2461, 2531, 2687, 3007, 3171, 4348.

[a] Application of the PuReTaq Ready-To-Go PCR Beads system.

hamper acquisition of MIRU-VNTR data. However, longer delays might make it possible to accumulate cartridges and minimize the number of deliveries and costs.

We evaluated the impact of longer delays (8–31 days from Xpert testing) in a subset of 8 specimens with high bacterial load, all of which were analysed using the 15 and 24-locus MIRU-VNTR format (Table 4). The 3 remaining specimens, not included in the analysis until now, were eliminated from the study because they corresponded to low/medium bacterial loads that proved to be problematic even in the absence of a delay. First, focusing on the 15-locus MIRU-VNTR format, a complete MIRU-VNTR pattern was obtained in 4 samples processed 8–31 days after Xpert testing (although in 1 case it required the application of the bead adaptation in 4 additional loci to fulfil the MIRU pattern). Although the number of samples was small, the association between acquisition of a complete 15 locus MIRUtype and delay in processing the samples for MIRU-VNTR analysis was statistically significant (comparing delays shorter or longer than 8 days, Fisher's exact test p-value 0.029). This association is more patent with the more demanding 24 locus format, where a complete MIRUtype was obtained in only 3 out of 8 samples (Fisher's test p- value 0.009). These data lead us to recommend a best processing time shorter than 8 days.

Correlation is expected between the fingerprinting data obtained directly from Xpert remnants and those obtained from the corresponding cultures. We did not have access to the cultured isolates from this study in Mozambique. However, we compared the MIRU-VNTR patterns obtained directly from four Xpert tests in our institution in Spain with those obtained from their cultured isolates and they were identical (data not shown).

In the light of these results, we conclude that the final optimized procedure succeeded in obtaining full 24-locus MIRU-VNTR fingerprints on high-load specimens within one week since the Xpert test has been performed. Based on the improvement of performance when PuReTaq Ready-To-Go PCR beads system were included, we would recommend its expanded use.

As a collateral observation, 1 of the specimens included in this last evaluation led to a MIRU-VNTR pattern with double alleles in 13 loci (Table 4, sample 19), thus expanding the usefulness of our strategy for identification of mixed infections, which are expected in countries with a high TB burden. Furthermore, if strains in mixed infections involve different susceptibility patterns [15], the efficiency of therapy could be affected.

Our study is limited by its ability to identify transmission events owing to the small sample size. As expected, all cases were unclustered. We must accept a suboptimal sampling when Xpert is used as the reference material, as this test is not recommended for all patients in some high burden countries. The sampling based on the cases with an Xpert result available can never substitute the proper population-based sampling required in any epidemiological rigorous study, however, it can complement traditional culture-based approaches. As we were aware of the sample size limitations, our objective was not to run an epidemiology study but only to demonstrate, that MIRU-VNTR data could be obtained from Xpert remnants, in those settings where other higher-quality sampling efforts (systematic culture) are not feasible.

Our study highlights the potential usefulness of remnants from currently available commercial assays for molecular analysis beyond the applications proposed by the manufacturer. Our findings indicate that epidemiologically useful MTB fingerprinting patterns can be acquired in settings where culture is not available, as long as Xpert technology is available. The results from our pilot study paved the ground to apply this strategy, with wider sampling efforts, to identify transmission clusters in molecular epidemiology studies of TB. The broad global distribution of Xpert, which is supported by the WHO recommendation to incorporate it into the diagnostic algorithms of national TB control programmes, enables the performance of TB transmission studies in low-resource settings, many of which experience the highest burden of the disease.

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Appendix V

Chapter 2 Supplement

Mycobacterial genomic DNA from used Xpert MTB/RIF cartridges can be utilised for accurate second-line genotypic drug susceptibility testing and spoligotyping

Mycobacterial genomic DNA from used Xpert MTB/RIF cartridges can be utilised for accurate second-line genotypic drug susceptibility testing and spoligotyping

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Supplementary

Table 1: Primer details for amplification of fragments of the *gyrA* and *rss* regions using cartridge extract from the dilution series

Target region	Primer name	Primer sequence (5' – 3')	Annealing temperature (°C)	Expected size (bp)
gyrA	gyrA_F	TGACATCGAGCAGGAGATGC	62	344
	gyrA_F	GGGCTTCGGTGTACCTCATC		
rrs290	rrs290_F	TGCTACAATGGCCGGTACAA	62	290
	rrs290_R	CTTCCGGTACGGCTACCTTG		

Table 2: Results of MTBDR*plus* and MTBDR*sl* drug susceptibility testing using cartridge extract on clinical specimens stratified to smear status. MTBDR*plus* had high indeterminate results rifampicin-resistance false-positive rates for both smear positive and smear negative specimens. MTBDR*sl* had low indeterminate rates for both RIF-susceptible and RIF–resistant specimens. Smear negative specimens had higher rates of non-actionable results for MTBDR*sl*.

Xpert positive rifampicin-susceptible and -resistant cartridges*								
Smear-positive specimens				**Smear-negative specimens**				
MTBDR*plus* (n=37)		**MTBDR*sl* (n=43)**		**MTBDR*plus* (n=13)**		**MTBDR*sl* (n=23)**		**p-values†**
TUB-band positive 32/37 (86)		**TUB-band positive 42/43 (98)**		**TUB-band positive 10/13 (77)**		**TUB-band positive 21/23 (91)**		
Rifampicin (%)		**Fluoroquinolones** (%)		**Rifampicin** (%)		**Fluoroquinolones** (%)		p=0.01
Susceptible	0/32 (0)	Susceptible	41/42 (98)	Susceptible	0/10 (0)	Susceptible	17/21 (81)	
Resistant	32/32 (100)	Resistant	1/42 (2)	Resistant	10/10 (100)	Resistant	1/21 (5)	
Indeterminate	0/32 (0)	Indeterminate	0/42 (0)	Indeterminate	0/10 (0)	Indeterminate	3/21 (14)	
Isoniazid (%)		**Second-line injectables** (%)		**Isoniazid** (%)		**Second-line injectables** (%)		p=0.01
Susceptible	10/32 (31)	Susceptible	39/42 (93)	Susceptible	0/10 (0)	Susceptible	16/21 (76)	
Resistant	0/37 (0)	Resistant	2/42 (5)	Resistant	0/10 (0)	Resistant	1/21 (5)	
Indeterminate	22/32 (69)	Indeterminate	.1/42 (2)	Indeterminate	10/10 (100)	Indeterminate	4/21 (19)	
TUB-band negative				**TUB band-negative**				p=0.015
5/37 (14)		1/43 (2)		3/13 (23)		2/23 (9)		

* Table shows results from both Xpert positive RIF-susceptible and RI-resistant specimens. RIF-susceptible samples had MTBDR*plus* and MTBDR*sl* done. RIF-resistant specimens only had MTBDR*sl*. 37/56 (66%) RIF-susceptible specimens were smear positive; 13/56 (23%) were smear negative and 6/56 (11%) had no smear results. 6/29 (20%) of RIF-resistant specimens were smear positive while 10/29 (35%) were smear negative and 13/29 (45%) had no smear result.

Appendix VI

Chapter 3 Supplement

Accurate diagnosis of second-line drug resistant tuberculosis and *rpoB* cross-contamination risk assessment using extract from used Xpert MTB/RIF Ultra cartridges

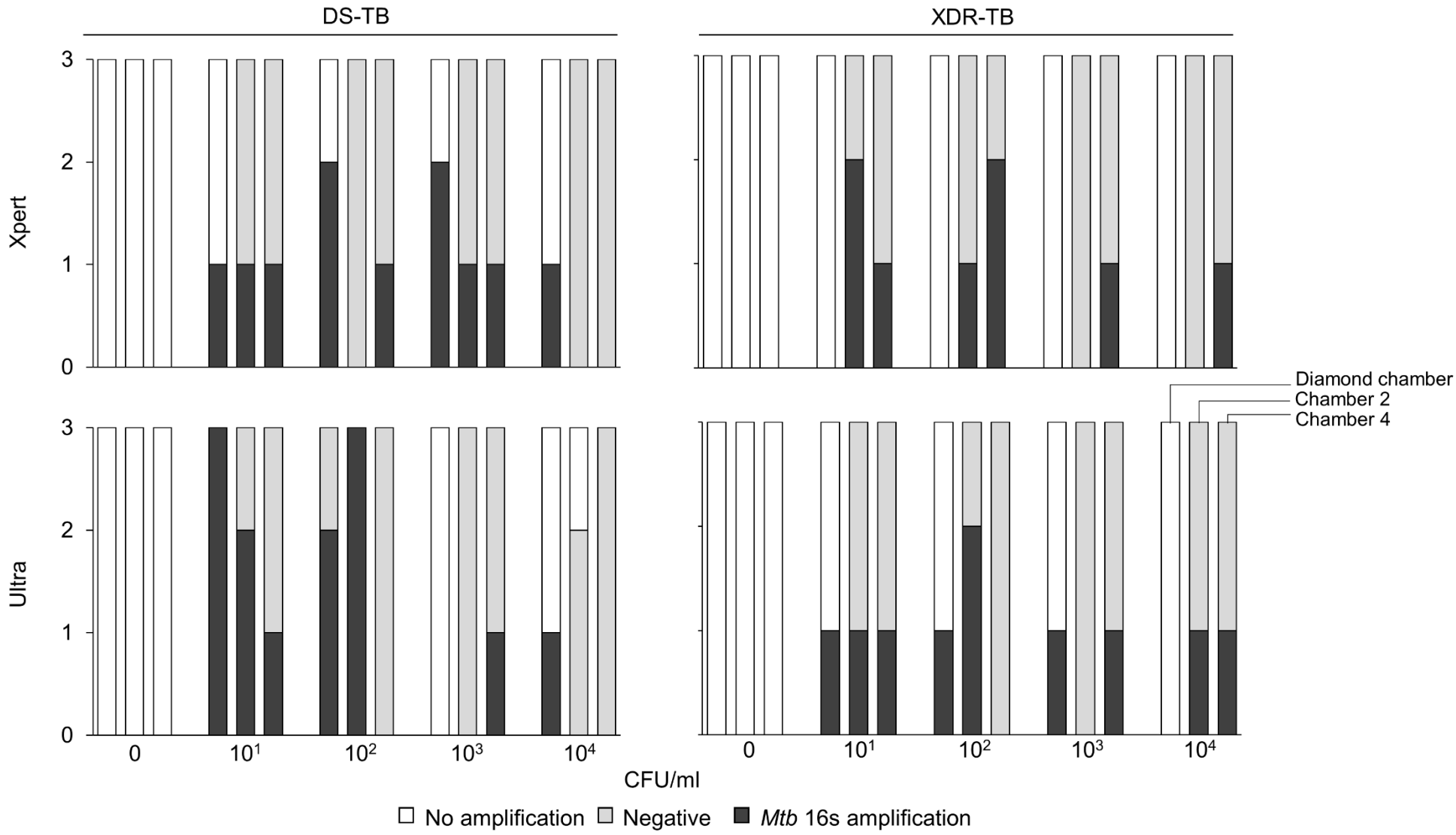
Supplement:

17 **Table S1**: V4 region *Mtb* specific 16S rRNA primers and thermocycling conditions[1]

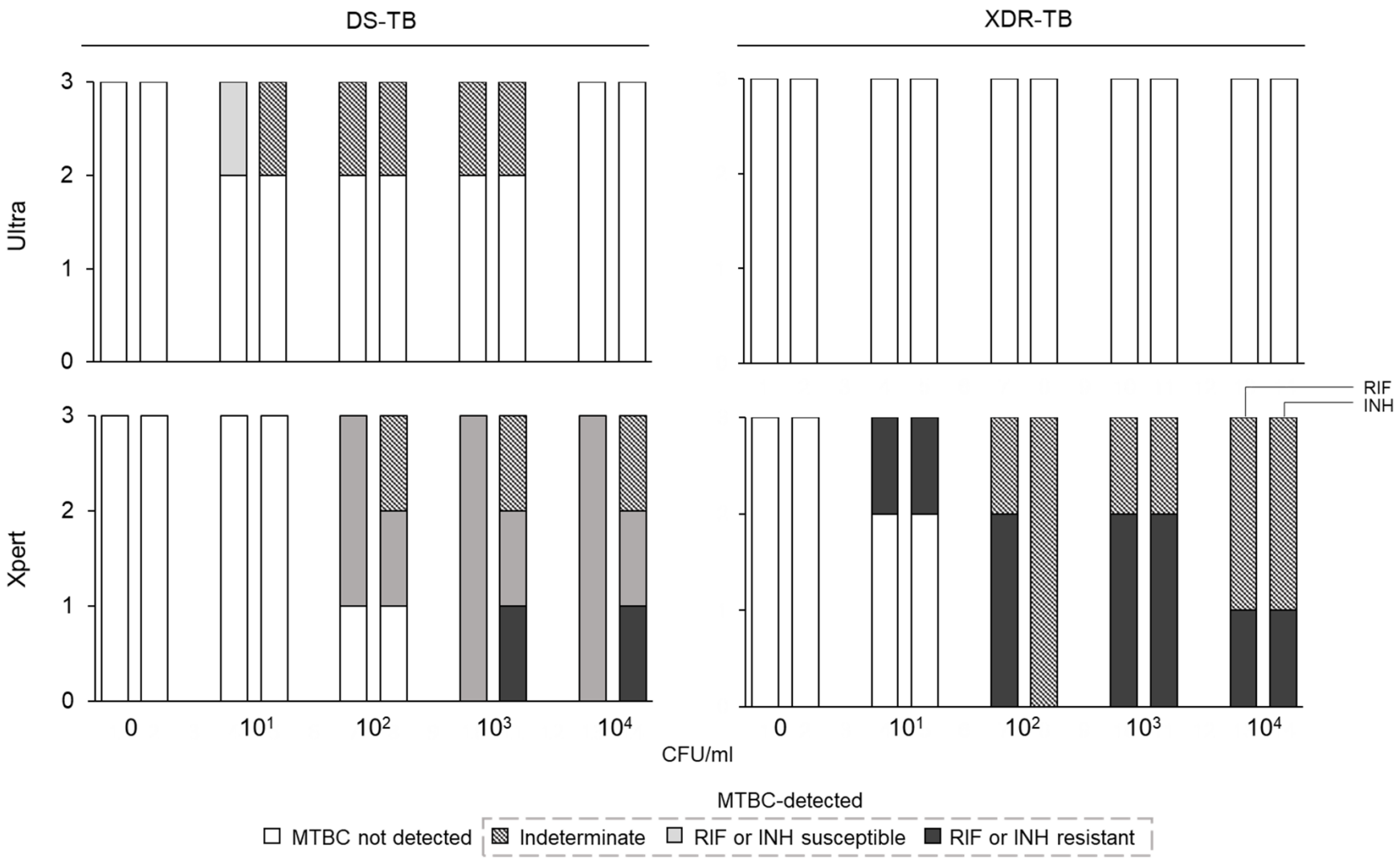
Mtb Forward primer	5'-GTGCCAGCAGCCGCGGTAA-3'
Mtb Reverse primer	5'-GGACTACCAGGGTATCTAAT-3'
Thermocycling conditions (Bio-Rad CFX-96 Real-Time PCR machine)	95°C for 5 minutes 35 cycles of 95°C for 5 seconds 60°C for 30 seconds.

18

19 **Figure S1**



20 **Figure S2:**



Ultra and Xpert cartridge structure and design

First, sample reagent is mixed with the specimen and 2 ml pipetted into the cartridge [Chamber 1 (C1); ~500 µl typically remaining after test completion]. The cartridge lid is then closed before placement in the GeneXpert machine and the test started. A plunger is automatically inserted into the cartridge centre. This engages a barrel to sequentially draw buffers and reagents into the chambers[2,3]. The plunger first draws the sample mixture into the cartridge base where bacilli (not necessarily alive or intact) are trapped on a filter prior to DNA extraction (Figure 2A)[4]. Wash fluid in Chamber 2 (C2, ~3 ml remaining), is then drawn over the filter and waste is collected in Chamber 3 (C3, ~5 ml remaining). While this wash can mostly (but not completely) remove small DNA fragments, it does not remove large DNA, especially if debris-associated[4-6]. Liquid reagent is then drawn from Chamber 4 (C4, ~500 µl remaining) and a sonic horn applied to lyse bacteria and release DNA[7]. DNA is then drawn into Chamber 5 (C5, no volume remaining) and mixed with reaction beads. This mixture is channelled into the reaction chamber (diamond protrusion at the back of cartridge where dCE is drawn from) where thermocycling and PCR amplification takes place[7].

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Appendix VII

Chapter 5 Supplement

Improving the utility of tuberculosis diagnostic tests for monitoring response to treatment

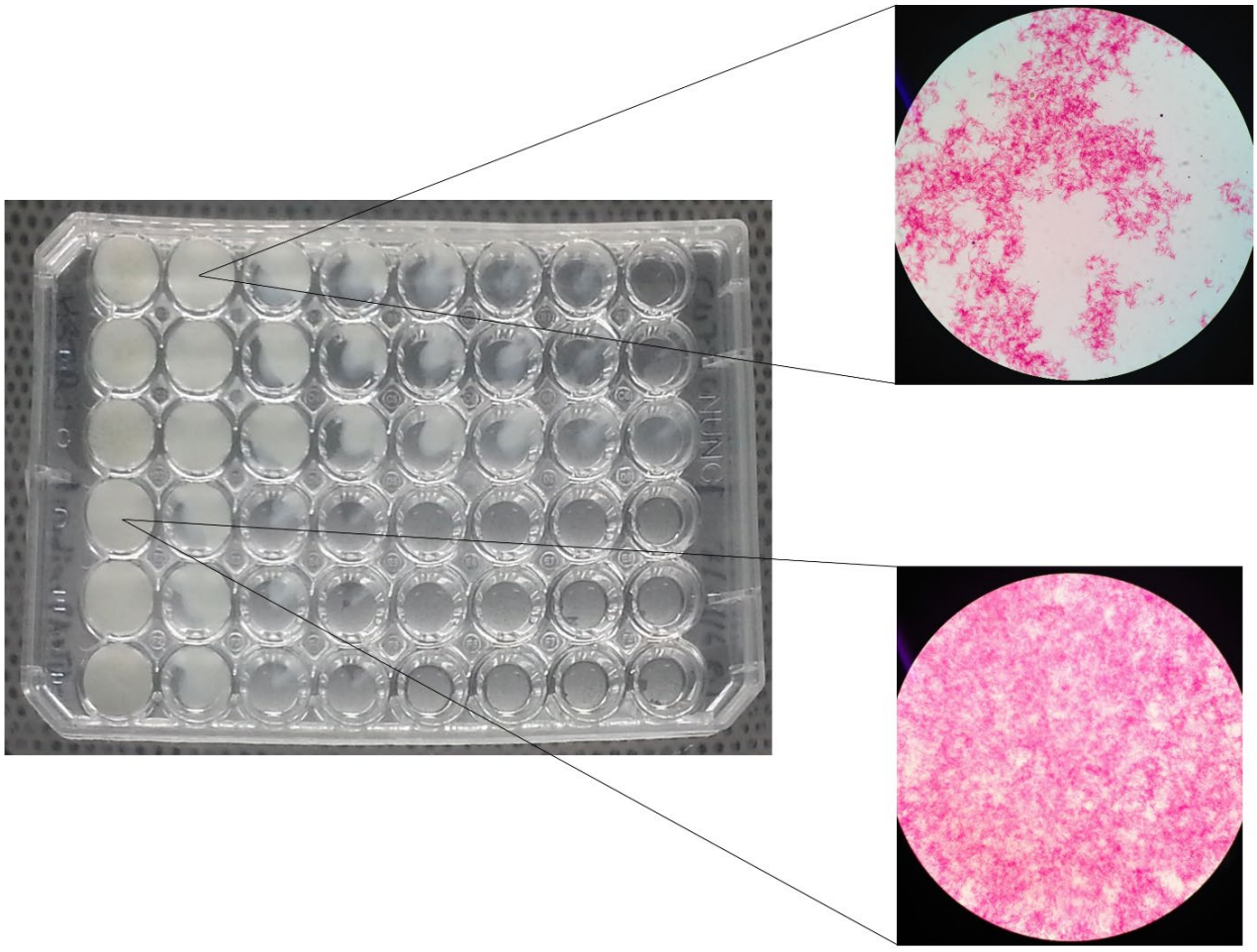
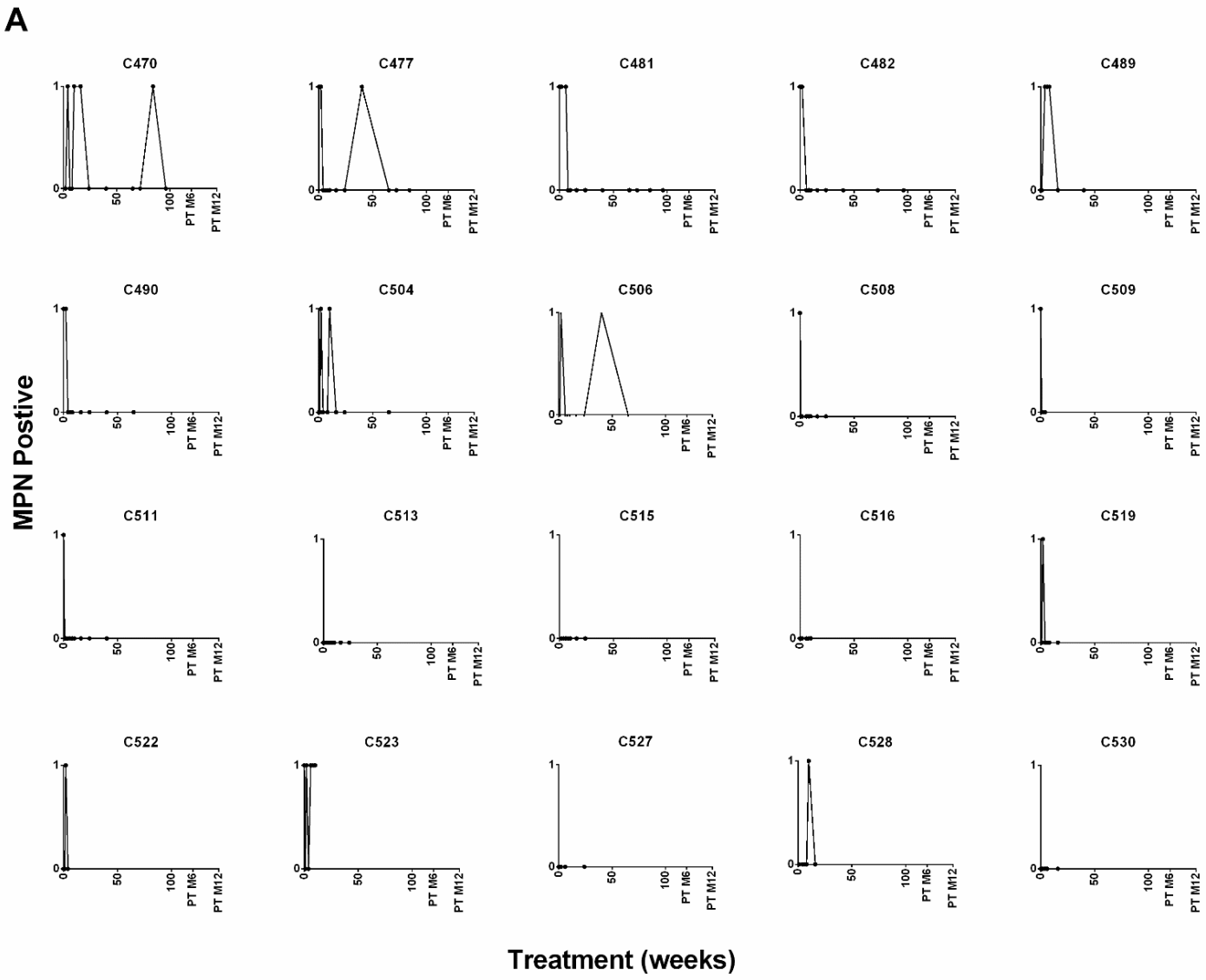


Figure S1: Example of a positive MPN plate from a study participant. Each positive well is confirmed for acid fast growth by plating on Blood agar plates as well as Ziehl-Neelsen acid fast staining as can be seen using wells A2 and D1 as examples.



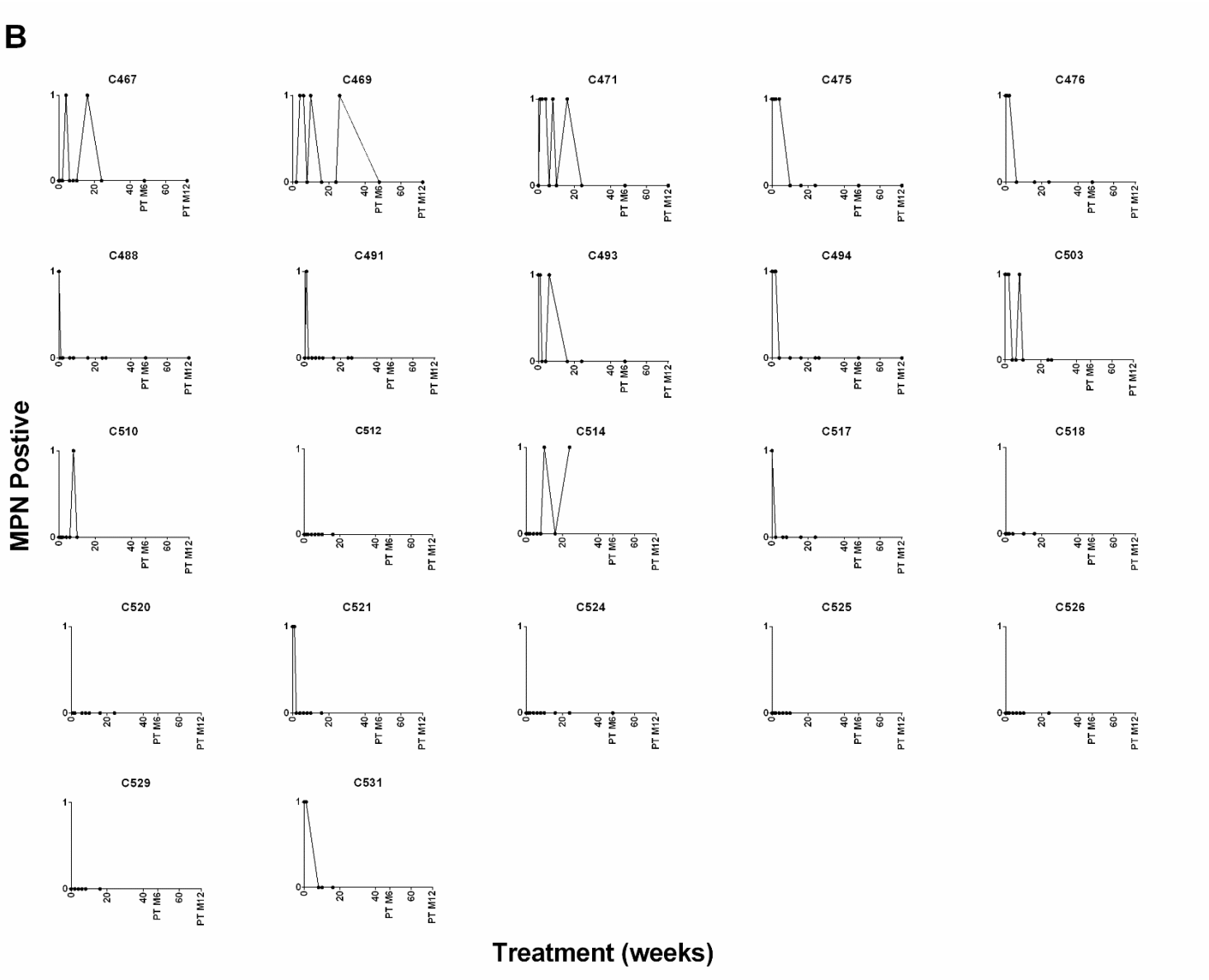


Figure S2: Standard Xpert (right) and vPCR (PEMAX treated) Xpert (left) C_{Tmin} values per patient for **[A]** The routine arm and **[B]** the intervention arm. C_{Tmin} values did not differ significantly between Xpert and vPCR throughtout treatment.

Figure S3:

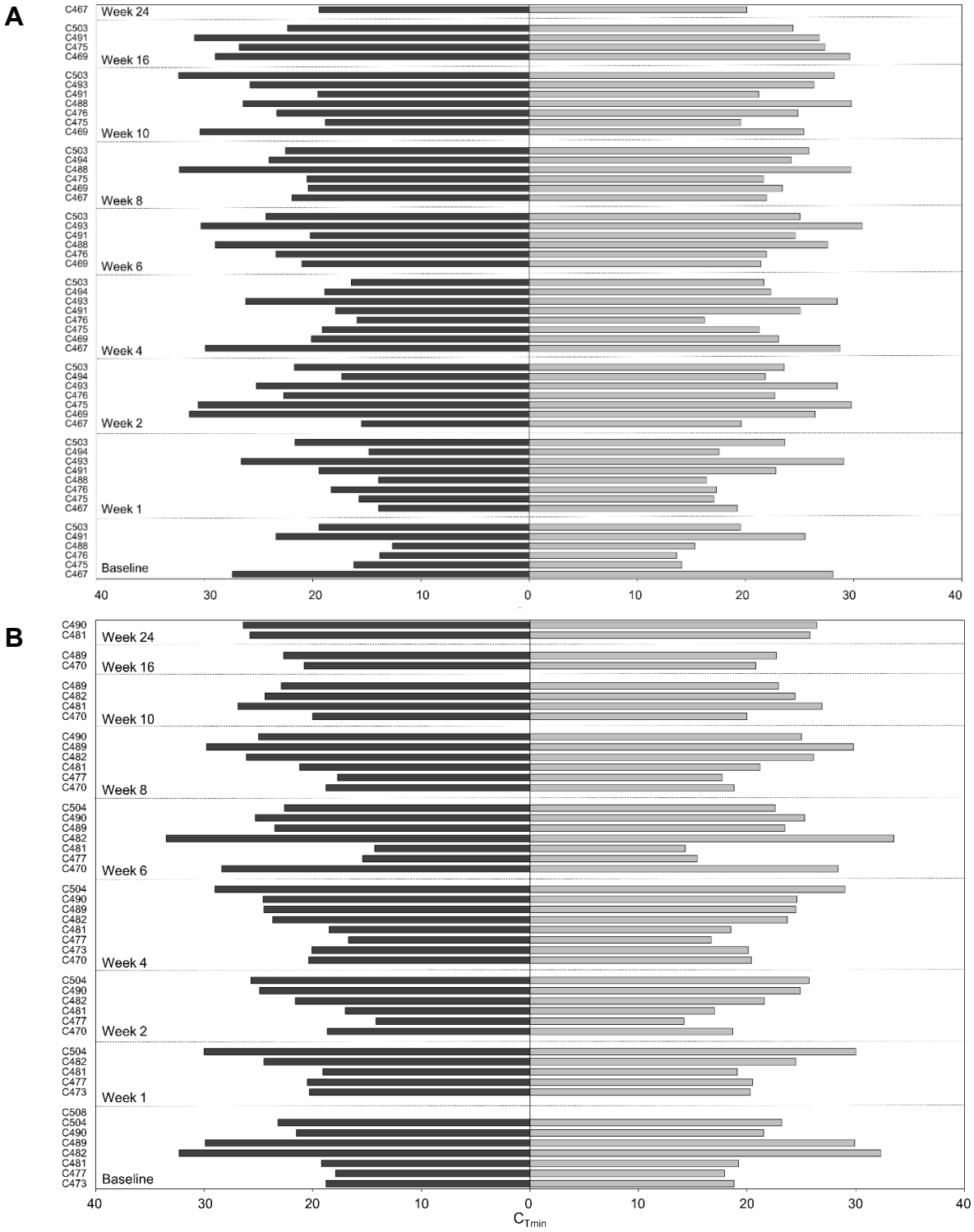


Figure S3: Patient Most Probable Number (MPN) assay results across treatment and post treatment month 6 (PT M6) and month 12 (PT M12) for patients on the **[A]** Routine treatment arm and the **[B]** Intervention treatment arm. There were no significant differences between the Xpert and vPCR-Xpert, indicating vPCR treatment did not reduce detection of non-viable bacilli.

Table 1: Inclusion and exclusion criteria of the Phase III clinical drug trial (NExT) from which sputum samples were sourced for this study.

Inclusion criteria:	Subjects are required to meet ALL of the following inclusion criteria to participate: • Newly diagnosed culture and/or GeneXpert positive pulmonary TB. • Rifampicin resistance detected using at least two susceptibility testing assays (GeneXpert, HainMTBDR*plus* or phenotypic) using a sputum sample during screening • Provide written informed consent prior to all trial-related procedures including HIV testing. • Male or female aged 18 years and older. • Body weight between 40 and 90 kg, inclusive. • Women of non-childbearing potential or participants of either sex who are using or willing to use effective methods of birth control.
Exclusion criteria:	Subjects will be excluded from participation if they meet ANY of the following criteria: • A subject who in the opinion of the investigator is unlikely to cope with regular visits to the trial site either because of travel constraints, or because of drug or alcohol abuse, or other reason. • Known at screening to have XDR-TB or pre-XDR-TB (i.e. fluoroquinolone or second-line injectable drug (SLID) resistance i.e. to capreomycin, amikacin and kanamycin). • Previous history of treatment for MDR-TB or XDR-TB or previous treatment with bedaquiline. • Currently on MDR-TB treatment for more than 2 weeks. • Any subject with a Karnofsky score $\leq$ 50. • Known allergy to any of the trial drugs or related substances. • Having participated in other clinical studies within 8 weeks prior to trial start where investigational agents were used that may potentially impact current trial outcome. • Presence (or evidence) of symptomatic neuropathy grade 3 or higher. • Epilepsy where drugs prolonging QT interval are used. • Participant who is pregnant, breast-feeding (and not willing to stop), or planning to conceive a child within 6 months of cessation of treatment. • Incompatibility between microbiological and clinical/ radiological findings (i.e. where the clinical and/or radiological findings are discordant with microbiological testing suggesting laboratory contamination). • Participants with ECG abnormalities, in particular QT prolongation. • Any pre-existing laboratory abnormality which in the opinion of the investigator will place the participant at risk (see detailed protocol for grade of abnormality).

	• Specific prior or concurrent medication/treatments (see detailed protocol). • Rifampicin-monoresistant TB (this criteria was amended during the trial and Rifampicin-monoresistant participants were included) • Fluoroquinolone and/or SLID resistance. Although in South Africa, the standard of care does not single out MDR-TB with fluoroquinolone or aminoglycoside resistance at initiation of MDR-TB treatment, in this study the Hain MTBDR*sl* line probe assay (LPA) will be used on the sputum sample to exclude any pre-XDR and XDR cases from participation in the study (results from the LPA and phenotypic DST testing on the isolate will be available 3-6 weeks later). • All inclusion and no exclusion criteria must be met prior to enrolment and randomisation. Whenever the investigator has reason to suspect that there might be a health problem (other than TB) participation should only be considered after discussing the case with the medical monitor. Note: Subjects who are currently on, or have previously been on drug-sensitive TB treatment are not excluded from participation. Post-randomisation exclusion criteria: Fluoroquinolone and/or SLID resistance detected on DST using the isolate. • Note: A woman who falls pregnant during the treatment phase of the trial will not be excluded but will be counselled regarding potential termination of pregnancy.

Appendix VIII
Chapter 6 Supplement

Use of a modified face mask and liquid-based cough aerosol sampling to measure the infectiousness of drug-susceptible and drug-resistant tuberculosis patients

Table S1: Results of duration study including negative results. Of 10 patients tested, 4/10 (40%) were gMask-positive of which no significant difference in time to positivity (TTP). There were 2 patients that were gMask negative but CASS positive, indicating that gMask may miss some patients CASS detects and vice versa.

PID	Time (h)	Aerosol culture result (TTP)	Culture (TTP)	CASS result
M014	1	P (14)	P (4)	P
	3	P (27)		
	6	P (18)		
M016	1	P (22)	P (5)	P
	3	P (20)		
	6	P (17)		
M017	1	P (23)	P (9)	N
	3	P (3)*		
	6	P (21)		
M019	1	N	N/A+	N
	3	N		
	6	P (24) p=0.47		
M011	1	N	P (7)	P
	3	N		
	6	N		
M012	1	N	N	N
	3	N/A		
	6	N/A†		
M013	1	N	P (13)	N
	3	N		
	6	N		
M015	1	N	P (12)	P
	3	N		
	6	N		
M018	1	N	N	N
	3	N		
	6	N		
M020	1	N	N	N
	3	N		
	6	N		

+ Patient was ill and could not produce sputum. † Patient was ill and did not wear mask for the full 3 and 6 hours.